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This thesis, AN OXIDIZED FAT CONTAINING DIET DECREASES WEIGHT GAIN BUT INCREASES ADIPOSITY IN MICE FED A LOW FAT DIET, by Mary Schneider was prepared under the direction of the Master's Thesis Advisory Committee. It is accepted by committee members in partial fulfillment of the requirements for the degree Master of Science in the College of Health and Human Sciences, Georgia State University.

The Master's Thesis Advisory Committee, as representatives of the faculty, certifies that this thesis has met all standards of excellence and scholarship as determined by the faculty.

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ABSTRACT

AN OXIDIZED FAT CONTAINING DIET DECREASES WEIGHT GAIN BUT INCREASES ADIPOSITY IN MICE FED A LOW FAT DIET

by
Mary Schneider

Introduction: Fast and convenience foods are abundant, relatively inexpensive, and accommodating to the fast-paced lifestyle of many Americans. One popular method of cooking used by many fast food establishments is deep-fat frying. Soybean oil is commonly used for frying and is rich in polyunsaturated fatty acids (PUFA) such as linoleic acid (LA). When soybean oil is used for deep-fat frying, LA becomes oxidized (Ox-LA). Endogenous Ox-LA has the capacity to be a ligand to peroxisome proliferator-activated receptor gamma (PPAR- γ), a nuclear transcription factor that regulates adipocyte maturation. It is not yet known whether or not dietary Ox-LA has the same capacity with respect to PPAR- γ . Considering the fact that dietary oxidized lipids are abundant in the typical American diet, it is important to know if they regulate weight gain and especially adipose tissue mass. In this study, we investigate the effects of fresh and heated soybean oil on weight gain and adiposity in mice fed isocaloric low fat diets.

Methods: Soybean oil was heated on a hot plate, under a hood, at 190°C for three hours. Fresh soybean oil served as the source of unoxidized oil (Unox-oil) and the heated oil served as the source of oxidized oil (Ox-oil). Both the Ox-oil and Unox-oil were incorporated into a low-fat (10% of calories) mouse chow by Research Diets, Inc. (New Brunswick, NJ). Sixteen C57BL/6J mice were divided into two groups and fed low fat diets with Ox-oil (low fat oxidized, LFO) or with Unox-oil (low fat unoxidized, LFU). Another group of 8 mice were pair fed to the LFO group with the Unox-oil containing chow (PLU). Mice in the LFO and LFU groups were fed ad libitum and known amounts of fresh food were added to the cages every three days. Leftover food was weighed.

Body weights were measured once a week. After 16 weeks mice were euthanized and epididymal white adipose tissue (EWAT), retroperitoneal white adipose tissue (RWAT), inguinal white adipose tissue (IWAT), and intrascapular brown adipose tissue (IBAT) samples were collected, weighed and stored at -80°C until further analysis. Fat pads were homogenized and cytosolic and nuclear proteins were extracted by standard methods. These extracts were subjected to Western blotting to determine the amount of PPAR- γ in the cytosol and nuclear compartments of the fat pads. Differences in group means were analyzed by Mann Whitney U test. Comparisons were considered statistically significant at a p-value of < 0.05 .

Results: Final mean body weights were significantly different when comparing the mice in the LFU group to the pair fed mice (PLU) (mean \pm SD; 29.52 ± 1.09 grams (g) and 26.85 ± 1.44 g, respectively; $p < 0.05$). Mice fed a low fat diet consisting of Ox-oil (LFO) had a final mean body weight of 27.88 ± 2.03 g. Mice in the LFU group gained significantly more weight on average than did mice in the LFO or PLU groups (mean \pm SD; 8.86 ± 1.37 g, 7.10 ± 1.47 g, and 5.71 ± 1.13 g, respectively). Although mean food intakes were not significantly different between any of the three groups, the average food intake was greatest for the LFU mice in comparison to the LFO and the PLU mice (mean \pm SD; 20.65 ± 0.09 g/week, 18.40 ± 0.05 g/week, and 18.38 ± 0.19 g/week, respectively). Feeding efficiency (g of weight gain/g of food consumed) was the highest in the LFU mice compared to the PLU mice (mean \pm SD; 0.031 ± 0.005 g/g and 0.022 ± 0.004 g/g) and this difference was statistically significant. The LFO mice gained less weight per gram of food consumed than did the LFU mice (mean \pm SD; 0.028 ± 0.006 g/g). Mean weights of all fat pads in the LFO group were significantly greater than those of the LFU and PLU mice (mean \pm SD; 0.329 ± 0.109 g, 0.199 ± 0.055 g, and 0.219 ± 0.041 for EWAT, 0.091 ± 0.039 g, 0.050 ± 0.026 g, and 0.051 ± 0.017 for RWAT, 0.221 ± 0.065 g, 0.135 ± 0.053 g, and 0.144 ± 0.038 for IWAT, and 0.079 ± 0.012 g, 0.055 ± 0.013 g, and 0.062 ± 0.011 for IBAT, respectively). PPAR γ protein in the cytosol of EWAT fat pads was analyzed and quantified in comparison to the amount of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; loading control) present. Mean PPAR γ /GAPDH ratios for LFU mice was 0.226 ± 0.082 , for LFO mice was 0.264 ± 0.122 , and for PLU mice was

0.234 ± 0.108. Mean PPAR γ :GAPDH ratios were not significantly different between any of the groups.

Conclusion: It appears that the consumption of oxidized oil caused a significant decrease in weight gain and food intake (although not significant) and a significant increase in fat pad mass in mice compared to those consuming a diet with unoxidized oil. The lack of difference in the amount of PPAR γ among the three groups of mice suggests that the changes in weight gain and fat pad mass among the oxidized oil consuming animals is not mediated through regulation of PPAR γ protein. To our knowledge, ours is the first study to report that mice consuming a low fat diet inclusive of dietary oxidized lipids exhibit greater adiposity than do mice consuming a low fat diet consisting of unoxidized lipids.

AN OXIDIZED FAT CONTAINING DIET DECREASES WEIGHT GAIN BUT
INCREASES ADIPOSITY IN MICE FED A LOW FAT DIET

By
Mary Schneider

A Thesis Submitted to the Graduate Faculty of Georgia State University in Partial
Fulfillment of Requirements for the Degree

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ABBREVIATIONS

13-HODE: 13-hydroxy linoleate

13-HPODE: 13-hydroperoxy linoleate

BCA: bicinchoninic acid

°C: degrees Celsius

CD: conjugated dienes

DAR: Georgia State University Department of Animal Resources

DTT: DL-dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

EWAT: epididymal white adipose tissue

g: gram

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

HDAC4: histone deacetylase 4

HEPES: 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid

HF: high fat

HFO: mice fed a high fat diet consisting of oxidized linoleic acid

HFU: mice fed a high fat diet consisting of unoxidized linoleic acid

HIP: Hexane Isopropanol

HRP: horseradish peroxidase

IBAT: intrascapular brown adipose tissue

IWAT: inguinal white adipose tissue

kcal: kilocalories

LA: linoleic acid

LDL r -/-: low-density lipoprotein receptor knockout

LFO: mice fed a low fat diet consisting of oxidized linoleic acid

LFU: mice fed a low fat diet consisting of unoxidized linoleic acid

LOH: lipid hydroxides

LOOH: lipid hydroperoxides

LPO: lipid hydroperoxides

MDA: malondialdehyde

mRNA: messenger RNA

nm: nanometers

NaCl: sodium chloride

NaF: sodium fluoride

Na₂MoO₄: sodium molybdate

Na₃VO₄: sodium orthovanadate

NP-40: Tergitol® solution

One-Way ANOVA: One-Way Analysis of Variance

Ox-LA: oxidized linoleic acid

Unox-LA: unoxidized linoleic acid

PAGE: polyacrylamide gel for electrophoresis

PBS: phosphate-buffered saline

PHU: mice pair fed to those mice fed a high fat diet consisting of oxidized linoleic acid

PLU: mice pair fed to those mice fed a low fat diet consisting of oxidized linoleic acid

PPAR- γ : peroxisome proliferator-activated receptor gamma

PUFA: Polyunsaturated fatty acid

PVDF: polyvinylidene fluoride

rcf: relative centrifugal force

RSB: Georgia State University's Research Support Building

RWAT: retroperitoneal white adipose tissue

SD: standard deviation

SDS: sodium dodecyl sulfate

SPSS: Statistical Package for the Social Sciences

TZD: thiazolidinedione

TG: triglycerides

TBARS: thiobarbituric acid reactive substances

μ L: microliters

CHAPTER I

INTRODUCTION

Obesity has rapidly advanced in prevalence and is currently a major health risk in the United States (1-3). Fast and convenience foods are abundant, relatively inexpensive, and accommodating to the fast-paced lifestyle of many Americans. Unfortunately, these foods are often calorie dense, rich in fats, and processed or refined such that nutrient profiles are altered (2, 10). A healthy individual consuming a diet that is high in fat, regardless of the source of the fat, is generally believed to be at greater risk for increased adiposity than is a healthy individual consuming a diet that is low in fat, provided that the two diets are isocaloric. It is not well established, however, whether or not individuals consuming low fat diets can expect differing effects on adiposity or total body weight regulation depending upon the type of fat consumed. Could the source of dietary fat increase the risks for unhealthy patterns of adiposity or lipotoxicity in an individual who consumes a diet that is low in total fat?

Deep-fat frying is one form of cooking used by many fast food establishments. Deep-fat frying involves heating oil at high temperatures in the presence of oxygen. This results in the breakdown of the triglycerides present in the oil and the formation of volatile and nonvolatile oxidation products (10). In previous research conducted by Penumetcha et al., the effects of dietary oxidized versus unoxidized lipids on atherogenesis in mice were studied (4-6). Surprisingly, those mice fed diets that

incorporated oxidized linoleic acid consumed less food and gained less weight in comparison with the mice fed unoxidized linoleic acid. This occurred in spite of the fact that the diets were isocaloric and physical activity levels were similar. Considering the fact that dietary oxidized lipids are abundant in the typical American diet, it is important to understand the mechanisms of the effects of dietary oxidized lipids on weight regulation (2).

CHAPTER II

REVIEW OF LITERATURE

Cooking oils, such as vegetable oil blends, are used for deep frying. Vegetable oil that is largely or entirely composed of soybean oil is especially popular for frying. Soybean oil, along with safflower and sunflower oils, is rich in polyunsaturated fatty acids (PUFA). Specifically, the majority of soybean oil is composed of the omega-6 PUFA known as linoleic acid (LA). Oils are heated at very high temperatures for extended periods of time to produce deep fried foods such as French fries. Cooking in this manner, even for a short period of time, encourages the oxidation of triglycerides. Oxidized lipids consist of molecules that include highly reactive lipid hydroperoxides (LOOH) and lipid hydroxides (LOH), triglyceride polymers and dimers, and aldehydes (7-10).

Studies by Penumetcha et al. affirmed the role of oxidized linoleic acid (Ox-LA) in advancing atherogenesis and in contributing to the development of atherosclerosis (4-6). Unexpectedly, the authors also observed what seemed to be a correlation between a high fat, high cholesterol (HF) diet containing oxidized linoleic acid (Ox-LA) and adipose tissue specific gene expression (specifically, leptin). In unpublished work, Penumetcha explained how feeding low-density lipoprotein receptor knockout (LDL r -/-) mice Ox-LA as part of a HF diet yielded significantly higher levels of circulating plasma leptin, decreased food consumption and diminished weight gain in comparison with the animals fed medium fat and low fat diets with or without Ox-LA. Knowing that

endogenous Ox-LA has the capacity to be a ligand to peroxisome proliferator-activated receptor gamma (PPAR- γ) and that PPAR- γ is the nuclear receptor that largely dictates adipocyte maturation, Penumetcha et al. proposed further investigation into the interactions between ox-LA and PPAR- γ and the effects of such interactions on food intake and weight regulation in mice (11-13).

PPAR- γ is the molecular target of the thiazolidinedione (TZD) class of insulin-sensitizing drugs. TZDs have been shown to encourage the deposition of lipids into adipose tissue, as opposed to lipids being sequestered in muscle and liver (16, 17). Not only is lipid deposition in adipocytes enhanced, lipogenesis is stimulated. The action of building lipids in adipose tissue removes free fatty acids and triglycerides (TG) from the circulation. A high plasma TG concentration is linked to insulin resistance. Therefore, targeting PPAR- γ helps control glucose metabolism by increasing insulin sensitivity (15). Furthermore, it has been postulated that PPAR- γ has a more immediate effect on the insulin signaling pathway because it upregulates the actions of intracellular proteins responsible for glucose transport (17). Being strong PPAR- γ agonists, TZDs enhance insulin sensitivity but they have undesirable side effects that include increased adiposity and total body weight gain (14).

In this study, we investigated the effects on weight regulation and adiposity in mice fed isocaloric low fat and high fat diets containing either fresh oil or heated soybean oil. In contrast to previous studies by Penumetcha et al., which showed the impact of Ox-LA on atherosclerosis using LDL r^{-/-} mice, the present study used the C57BL/6J strain of mice. The C57BL/6J strain was a more appropriate mouse model for the present study because these mice are bred to become lean or obese depending upon the diet they

consume. For example, when consuming a high fat diet for a period of time, C57BL/6J mice become obese and experience obesity-related complications (i.e. hyperinsulinemia and hyperglycemia) as would humans on a similar diet. Methods and procedures included heating soybean oil, performing assays to analyze the primary and secondary oxidation products formed, incorporating the heated and fresh oils into mouse chow, performing the mouse study, and completing terminal procedures on all mice. Muscle, liver, and adipose tissue samples were collected during the terminal procedures. We have begun to analyze amounts of PPAR- γ protein present in the adipose tissue and will conclude this analysis in the near future. Our intention is to determine the expression of additional markers of adipocyte function (PPAR- γ messenger RNA (mRNA) and leptin) at a later date.

CHAPTER III

METHODS

Preparation for the mouse study: generating unoxidized and oxidized linoleic acid

Heating soybean oil

In order to produce the oxidized linoleic acid that was added to the mouse chow, soybean oil (Crisco® Pure Vegetable Oil) was heated on a hot plate, under a hood, at 190°C for three hours. An air compressor supplied a continuous flow of oxygen throughout the oil for the duration of the heating process. Careful maintenance of these conditions effectively oxidized the linoleic acid present in the soybean oil. Aliquots were taken at 1-hour intervals. Immediately upon retrieval, the aliquots were stored under nitrogen gas at -20°C.

Analysis for products of oxidation

Lipids were extracted from the heated soybean oil using a solvent system of Hexane Isopropanol (HIP) in a ratio of 3:2. Samples were homogenized in the HIP solution and the homogenate was filtered through a Buchner funnel. To evaporate the hexane, filtrates were dried under nitrogen gas. Filtrates were weighed and extracted fats were then used to determine the level of oxidation of the oil through measurement of the conjugated dienes (CD, primary product of oxidation), the lipid hydroperoxide content

(LPO, primary product of oxidation) and the amount of thiobarbituric acid reactive substances (TBARS, secondary products of oxidation) present.

1. The LPO assay

The LPO assay measures the amount of 13-hydroperoxy linoleate (13-HPODE) formed in the heated soybean oil (sample). A lipid hydroperoxide assay kit was purchased from the Cayman Chemical Company and the instructions enclosed in the kit were followed (19). Absorption of light at 500 nanometers (nm) was measured using a spectrophotometer. Standards were prepared and absorbance of the standards was measured alongside the samples. A standard curve was generated in order to extrapolate the unknown hydroperoxide concentrations of the samples.

2. The CD assay

To perform the CD assay, a sample of heated oil was diluted with hexane in a ratio of 1:3. A one milliliter volume of each diluted oil/hexane product was placed in a quartz cuvette and its absorbance, A , was measured at 234 nm. The extinction coefficient formula ($c = (A/\epsilon l)$, where the extinction coefficient, ϵ , is known to be 23,000 for Ox-LA at 234 nm, c is the unknown molar concentration of CD in the sample, and l represents the path length of the light or 1 centimeter) was used to obtain the combined amount of 13-HPODE and 13-hydroxy linoleate (13-HODE) present in the oxidized oil. By subtracting the values generated in the LPO assay from the values generated by the CD assay, the amounts of 13-HODE were obtained.

3. The TBARS assay

The TBARS assay is a reliable method of quantifying the malondialdehyde (MDA) present in a sample of lipid which has undergone peroxidation (18). Solutions

were prepared as indicated by the protocol and absorbance was measured at 540 nm (18). Levels of absorbance are directly related to the concentration of MDA in the sample. The absorbance levels of the experimental assay were charted against those of the standard assay in order to determine the concentration of MDA present.

Incorporation of heated oil into diets

Fresh soybean oil served as the source of unoxidized linoleic acid (Unox-LA) in the control diets. Both the Ox-LA and Unox-LA were incorporated into mouse chow by Research Diets, Inc. (New Brunswick, NJ). The low fat and high fat diets were isocaloric, with the low fat diets providing 3.75 kilocalories/gram (kcal/g) food and the high fat diets providing 4.1 kcal/g food. The low fat diets were composed such that 10% of the total calories came from fat, with the Unox-LA low fat diet having 0% of total fat calories contributed by oxidized lipids and the Ox-LA low fat diet having 3.33% of total fat calories contributed by oxidized lipids. The high fat diets were composed such that 45% of total calories came from fat, with the Unox-LA high fat diet having 0% of total fat calories contributed by oxidized lipids and the Ox-LA high fat diet having 15% of total fat calories contributed by oxidized lipids. Henceforth, diets incorporating Ox-LA may also be referred to as “modified” diets and diets incorporating Unox-LA may be referred to as “unmodified” diets.

Mouse Study

Animal model

Forty-eight C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were approximately 6-8 weeks old at the start of the study. Mice were grouped as shown in Scheme 1.

Animal care and maintenance

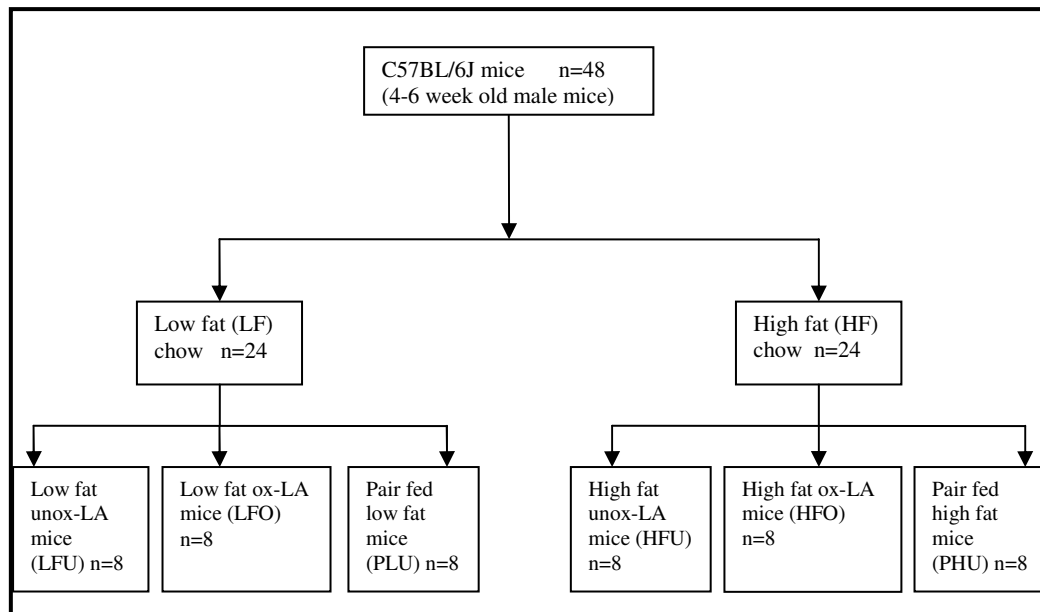
All animals in the study were housed in Georgia State University's Research Support Building (RSB). The mice were acclimatized for approximately two weeks before the experimental period began. For the duration of the acclimatization period, all mice were fed normal chow provided by the Georgia State University Department of Animal Resources (DAR). On the designated start date, normal chow was removed from all cages and replaced with the special diets described previously. Mice were given fresh chow at a minimum rate of 3 days/week. At each feeding, any remaining chow was collected and weighed. Animals were weighed at a minimum of once/week for total body weight in grams. A final body weight was taken just prior to euthanasia.

Feeding design

Mice were fed ad-libitum with the exception of the pair fed mice. Twenty-four of the 48 mice were fed a low fat diet while the remaining 24 mice were fed a high fat diet. Of the 24 mice consuming a low fat diet, 8 consumed a modified diet, 8 consumed an unmodified diet, and the final 8 were pair fed (explanation of the pair feeding technique follows). The same divisions were used for the 24 mice receiving the high fat chow. All

pair fed mice consumed an unmodified diet (8 consumed the low fat variety and 8 consumed the high fat variety). Although the pair fed mice were fed unmodified diets, they were fed in accordance with the food intake of those animals who received modified diets. In other words, we calculated the amount eaten by the mice fed a low fat modified (LFO) diet (recall these mice ate as they chose, with no restriction placed on the amount of food they could consume). After calculating the LFO group's ad-libitum intake, we weighed out approximately the same amount of food for the low fat pair fed mice (i.e. these mice *were not* fed ad-libitum). The same design was used for the mice pair fed to those mice maintained on a high fat modified (HFO) diet. The pair fed mice provided an additional measure of control over the results obtained from the LFO and HFO mice.

Scheme 1



Terminal procedure

The date of termination (euthanasia) was approximately 16 weeks from the initiation of the study. For the termination procedure, each mouse was anesthetized with

isoflurane. A cardiac puncture was performed in order to remove blood from the heart (up to 1 cubic centimeter). The collected blood was placed into a pre-prepared centrifuge tube, inverted several times, and centrifuged for ten minutes. Plasma was then removed from the centrifuge tube and stored in a clean tube at -80°C. Upon completion of the cardiac puncture, an incision into the abdominal cavity was made. This exposed the organs and tissue to be harvested. Biopsies of muscle and liver were made first. These samples were immediately wrapped in foil, flash frozen in liquid nitrogen, and subsequently placed in a -80°C freezer. Samples of epididymal white adipose tissue (EWAT), retroperitoneal white adipose tissue (RWAT), inguinal white adipose tissue (IWAT), and intrascapular brown adipose tissue (IBAT) were collected. These tissue samples required rinsing in phosphate-buffered saline (PBS) at once upon removal. After cleaning, rinsing, and blotting, the fat pads were weighed using a high-precision balance and weights were recorded. Pads were transferred to labeled pieces of aluminum foil and were wrapped, flash frozen in liquid nitrogen, and also placed in a -80°C freezer. Ultimately, all samples will be analyzed for the expression of PPAR- γ mRNA and the PPAR- γ protein. In addition, plasma samples will be analyzed for levels of plasma leptin.

Western Blotting

The harvested fat pads were homogenized in pre-chilled Dounce homogenizers filled with a hypotonic buffer solution (20mM HEPES pH 7.5, 5mM NaF, 0.1mM EDTA, and 1 mM Na₃VO₄) containing 0.01% NP-40 (hypotonic buffer #1). Suspensions were allowed to incubate on ice for 15 minutes followed by centrifugation for 10 minutes at

4°C and 8,500 rcf (~9,000 rpm). Supernatants (cytosolic fractions) were removed and stored at -80 °C. The remaining nuclear pellets were washed several times with 500 µL hypotonic buffer containing 0.5% NP-40 (hypotonic buffer #2), while special care was taken to avoid disturbance of the pellet itself. The buffer was removed and discarded. The process of washing and then discarding the buffer was repeated once more for each nuclear pellet. Each pellet was aspirated by pipette to a fresh tube filled with 500 µL hypotonic buffer #2, incubated on ice for 15 minutes, and centrifuged for thirty seconds at 14,000 rcf. The hypotonic buffer was discarded and pellets were re-suspended in a lysis buffer (20mM HEPES pH 7.5, 400mM NaCl, 20% glycerol, 0.1% EDTA, 10mM NaF, 10mM Na₂MoO₄, 1mM Na₃VO₄, and 10mM beta-glycerophosphate) containing 1mM DTT and Complete, Mini, EDTA-free protease inhibitor cocktail (catalog number 11 836 170 001; Roche Diagnostics Corporation, Indianapolis, IN). The suspensions were centrifuged for approximately three seconds and stored at -80 °C. Protein concentration of each sample was assessed using the bicinchoninic acid assay (BCA assay) performed using a BCA kit (product code BCA1 AND B9643; Sigma, St. Louis, MO). Absorbance was measured using a plate reader warmed to 37°C and set at a wavelength of 562 nm. The absorbance levels of the experimental assay were charted against those of the standard assay in order to determine how many µg of protein could be expected to be obtained from each µL of sample. For the Western blotting applications, approximately 50 µg protein from most samples were used. A SDS-10% polyacrylamide gel for electrophoresis (SDS-PAGE) served as the medium for fractionating proteins from samples. Samples, standard markers (Cruz Marker™ Molecular Weight Standards, sc-2035; Santa Cruz Biotechnology, Inc., Santa Cruz, CA,

and Page Ruler™ Pre-stained Protein Ladder, #SM0671; Fermentas, Inc., Glen Burnie, MD), a Western blotting positive control (3T3 L1 cell lysate: sc-2243; Santa Cruz Biotechnology, Inc.) and a control recombinant protein for PPAR γ (PPAR γ (6-105): sc-4546; Santa Cruz Biotechnology, Inc.) migrated through the SDS-PAGE by electrophoresis. The fractionated proteins were then transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). All membranes were immunoblotted with antibodies purchased from Santa Cruz Biotechnology, Inc. Probing for PPAR γ protein expression was performed using a 1:400 dilution of primary antibody (PPAR γ (H-100): sc-7196) detected by a 1:4,000 dilution of secondary antibody (goat anti-rabbit IgG HRP: sc-2030). As a control measure, membranes containing cytosolic samples were also immunoblotted for GAPDH using a 1:1000 dilution of primary antibody (GAPDH (6C5): sc-32233) detected by a 1:4,000 dilution of secondary antibody (goat anti-mouse IgG HRP: sc-2031). Membranes containing nuclear samples were immunoblotted for HDAC4 using a 1:1000 dilution of primary antibody (HDAC4 (A-4): sc-46672) detected by a 1:4,000 dilution of secondary antibody (goat anti-mouse IgG HRP: sc-2031). Blots were incubated in chemiluminescent reagents (Immobilon Western Chemiluminescent HRP substrate, catalog number WBKLS0100; Millipore) and digital images revealed signals emitted by the secondary antibody. Imaging was made possible through the use of AlphaEase FC (Fluor Chem 8800) software and a MultiImage™ Light Cabinet (Alpha Innotech Corporation, San Leandro, CA). Gels were analyzed using Quantity-One 1-D Analysis Software version 4.6.6 (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

Statistics were generated using SPSS version 16.0 (SPSS Inc., Chicago, IL).

Normally distributed variables were first analyzed using One-Way ANOVA followed by Bonferroni tests for multiple comparisons. Non-normally distributed variables were analyzed using the Kruskal-Wallis test followed by Mann-Whitney U tests. Comparisons were considered statistically significant with a p-value of < 0.05 . Results are shown as means and standard deviations (SD).

CHAPTER IV

RESULTS

Body weight

Although mean body weights of the mice in the low fat unmodified (LFU), low fat modified (LFO), and low fat pair fed (PLU) groups were not significantly different at the start of the study, the mice gained weight differentially and the final mean body weights were significantly different when comparing LFU to PLU (see Figure 1). At the study's conclusion, final mean body weights of the LFU, LFO, and PLU mice were 29.52 ± 1.09 grams (g), 27.88 ± 2.03 g, and 26.85 ± 1.44 g, respectively.

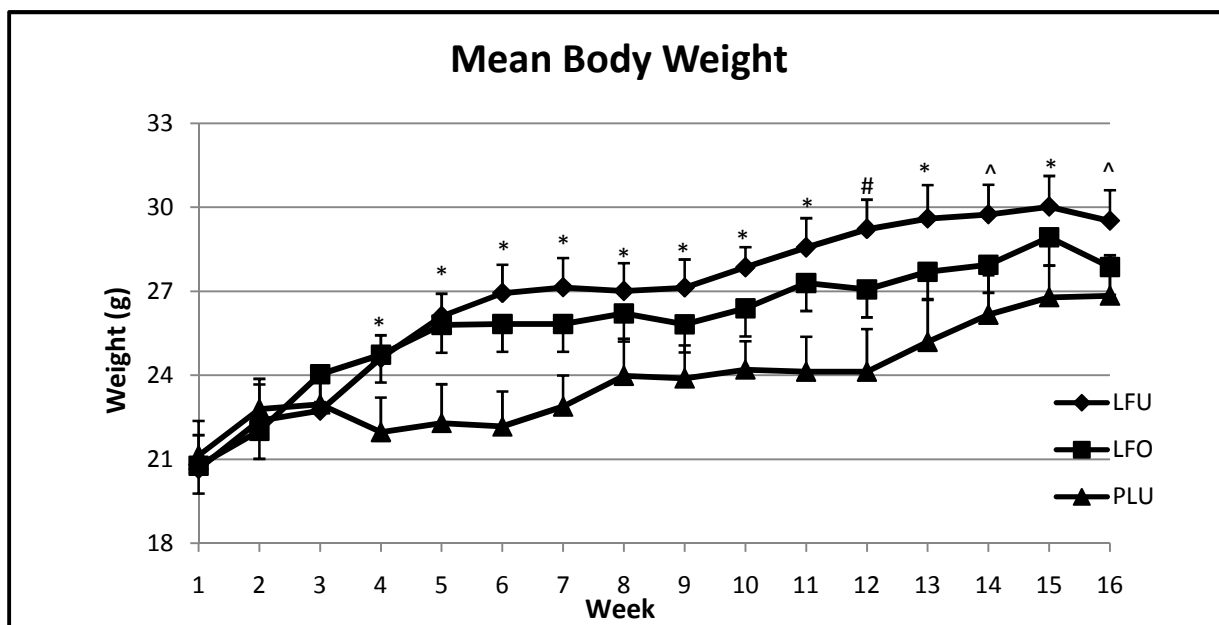


Figure 1. Mean body weights of LFU, LFO, PLU groups taken on a weekly basis. * $p < 0.05$ comparing groups LFU with PLU and LFO with PLU; # $p < 0.05$ comparing groups LFU with PLU, LFO with PLU, and LFU with LFO; ^ $p < 0.05$ comparing group LFU with PLU.

Weight gain

Similarly, mean weight gains were greatest for mice in the LFU group in comparison to those in the LFO and PLU groups. For the duration of the study, LFU gained an average of 8.86 ± 1.37 g, LFO gained an average of 7.10 ± 1.47 g, and PLU gained an average of 5.71 ± 1.13 g ($p < 0.05$ comparing groups LFU with LFO and LFU with PLU; see Figure 2).

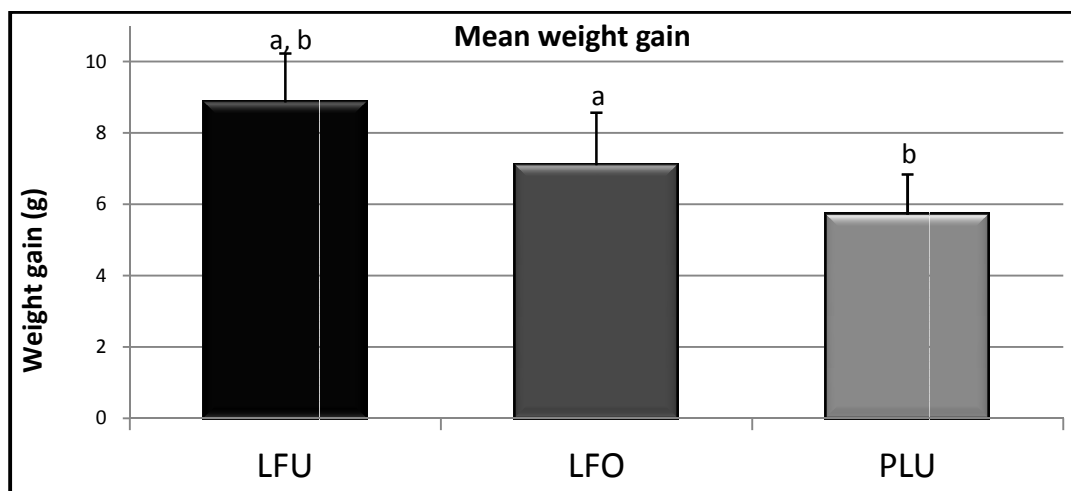


Figure 2. Mean final weight gains of LFU, LFO, and PLU groups. Groups with common letters above error bars are significantly different from one another ($p < 0.05$).

Food intake

Following the trends of the mean body weights and mean weight gains, the average food intake was greatest for the LFU mouse (20.65 ± 0.09 g/wk). The average LFO mouse consumed 18.40 ± 0.05 g/wk and the average PLU mouse consumed 18.38 ± 0.19 g/wk (see Figure 3). Mean food intake was not significantly different between any

of the groups.

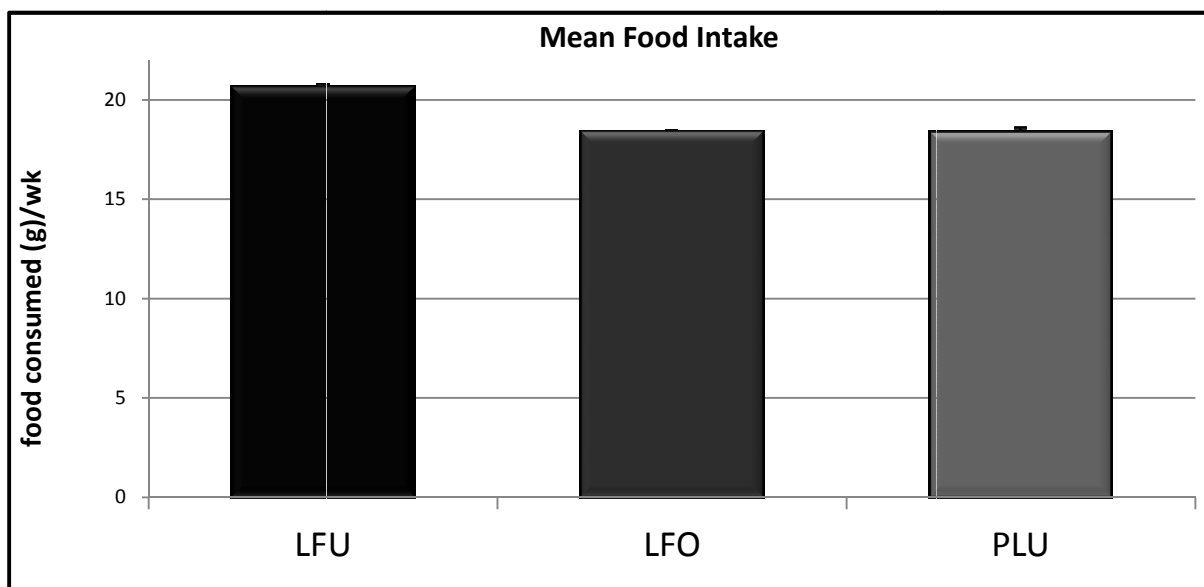


Figure 3. Mean food intakes for LFU, LFO, and PLU groups ($p > 0.05$).

Feeding efficiency

Feeding efficiency was calculated as the ratio of total grams of weight gained to total grams of food consumed. In spite of the fact that there was no significant difference in mean food intake, the LFO and PLU mice had to consume more grams of food than the LFU mice in order to gain one gram of weight, and this difference was significant between the LFU and PLU mice ($p < 0.05$; see Figure 4).

Fat pad weights

Mean weights of all fat pads were greatest in the LFO group. The weights were significantly different between the LFU and LFO groups, and between the LFO and PLU groups, but not between the LFU and PLU groups (see Figure 5).

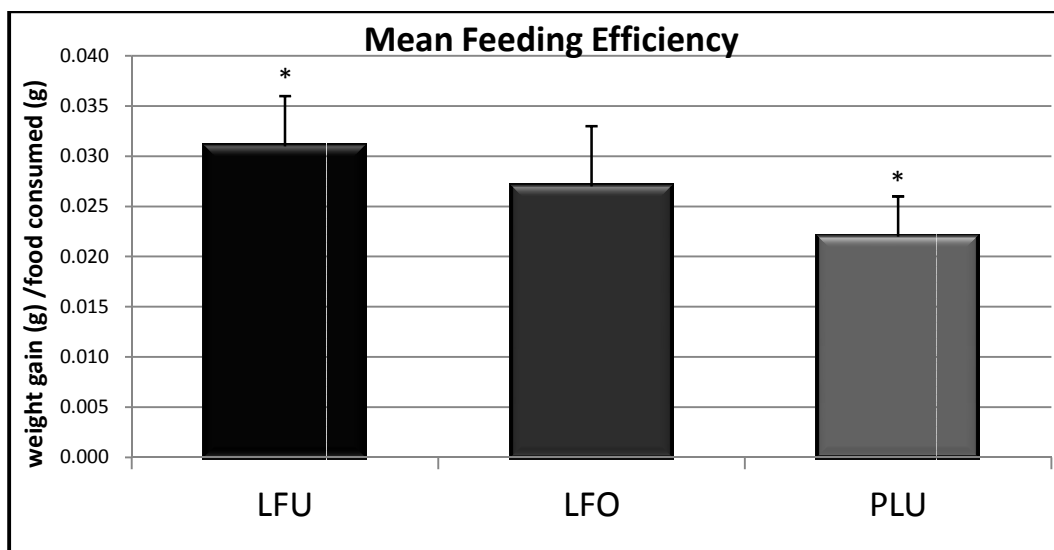


Figure 4. Mean feeding efficiencies for LFU, LFO, and PLU groups; * $p < 0.05$ comparing group LFU with PLU.

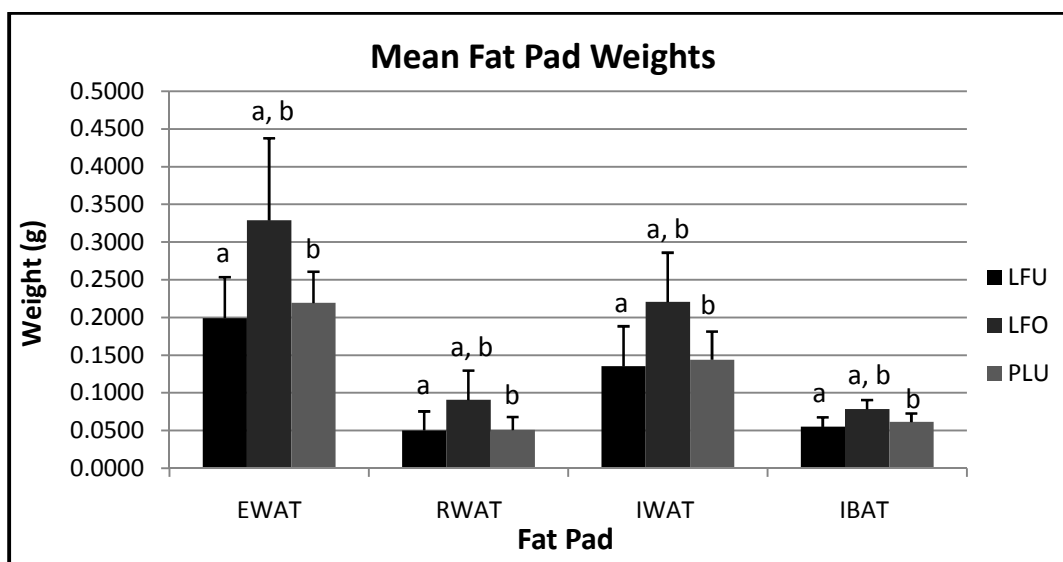


Figure 5. Mean fat pad weights of each of four fat pads harvested from LFU, LFO, and PLU groups. Groups with common letters above error bars are significantly different from one another ($p < 0.05$).

PPAR γ protein levels

PPAR γ protein in the cytosol of EWAT fat pads was analyzed and quantified in comparison to the amount of GAPDH present. Figure 6 shows the mean PPAR γ : GAPDH ratios for LFU, LFO, and PLU. The mean for LFU mice was 0.226 ± 0.082 , for

LFO mice was 0.264 ± 0.122 , and for PLU mice was 0.456 ± 0.595 . Mean PPAR γ : GAPDH ratios were not significantly different between any of the groups.

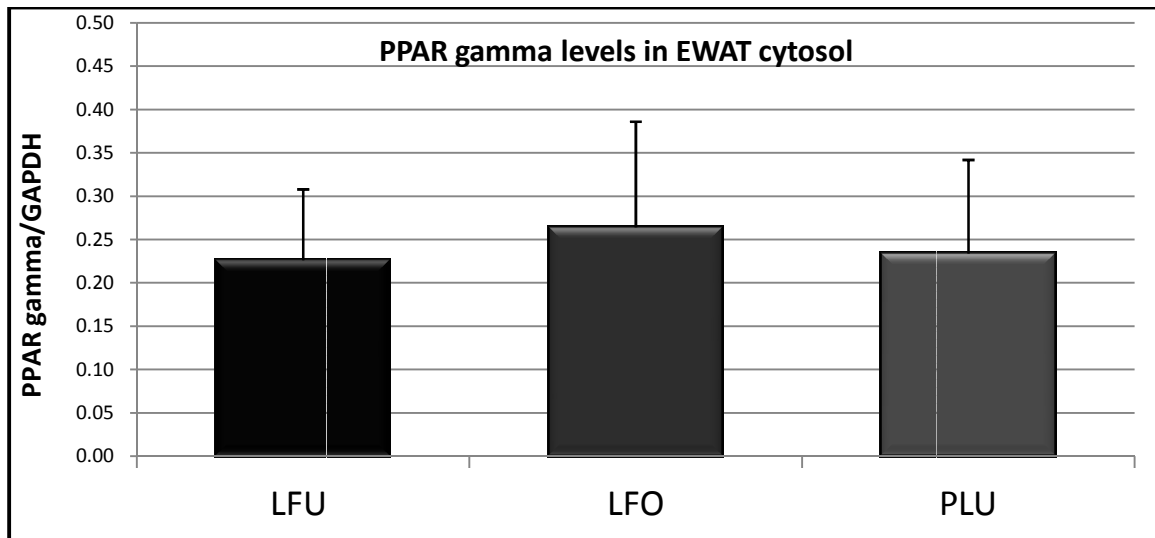


Figure 6. Expression of the PPAR gamma protein in EWAT cytosol samples.

CHAPTER V

DISCUSSION

Few studies have focused on the effects of dietary heated oils on rodents *in vivo*. To our knowledge, no study has examined thermally oxidized soybean oil and its impact on weight regulation and adiposity in mice. In the present study, the final measurements of mean body weights were taken after sixteen weeks, at which point body weights of LFU mice were significantly greater than those of PLU mice. Mean body weights taken after the twelfth week of the study showed significantly lower weights for LFO mice in comparison to LFU, but at no other point in the study were the body weights different between those mice fed the fresh oil versus those mice fed the heated oil. In contrast, studies published by Lopez-Varela, et al., and Garrido-Polonio, et al., reported final mean body weights of rats fed heated oil that were significantly lower than the body weights of rats fed fresh oil (20, 21). However, a few notable differences existed between these studies and the present study. In the previous studies, sunflower oil was used to fry potatoes for up to seventy-five separate frying operations before being incorporated into rodent diets. Also, the used oil contained oxidation products at a level of approximately fifteen times that of the unused oil and included products of advanced oxidation. In our study, the aim was to examine the effects of the primary products of oxidation of linoleic acid. Therefore, heating of soybean oil was performed in a single operation for three hours. This produced an experimental diet (LFO) consisting of approximately three

times more oxidation products in comparison to the control (LFU) diet. In our study, the lack of a significant difference in mean body weights of LFU mice in comparison to LFO mice was most likely due to the type of oil used, the method of heating oil in the absence of additional foods, or the extent of oxidation that occurred.

As the study progressed, it became apparent that the pair fed mice were gaining weight at a much slower rate than their counterparts (LFU mice). Since identical diets were fed to both the pair fed and LFU mice and food intakes were not significantly different, we explored other possible explanations for the marked difference in weight gain. Did the pair fed mice have increased physical activity levels, aggressive behaviors, grooming, or any other observable habits that could account for the greater energy expenditure in comparison to the LFU mice? To answer this question, on eight separate occasions and at varying times of day, myself and another researcher independently observed and compared the pair fed mice with non-pair fed mice. The mice were observed for all of the aforementioned behaviors and care was taken to minimize human interference with the mice as the observations were made. Upon review of the data, we could find no notable differences in any of the behaviors observed. Another possible explanation was that the pair fed mice may have been required to use more energy for retrieval of their food than did the non-pair fed mice. This thought occurred to us due to the manner in which mice were offered their food. The chow was placed in a hopper suspended into the cage, the mice could only gain access to their food by reaching up to the openings in the hopper and “pulling” the chow down, and a greater mass of food in the hopper forced the chow down and kept it in place near the openings of the hopper. Therefore, it seemed reasonable to consider the idea that those mice with less chow in

their hoppers would have to work harder to retrieve it in comparison to those mice with an abundance of chow in their hoppers. Recall that the amount of food given to the pair fed mice was a controlled, measured amount of food as opposed to the non-pair fed mice which were fed ad-libitum. The hoppers of cages containing pair fed mice were generally filled with less chow than were the hoppers of cages containing non-pair fed mice. This possible explanation for the leanness of the pair fed mice could not be upheld, however, because leanness was not necessarily seen in the cases where mice were housed individually and given only a few pellets at each feeding. To further explain, in both pair fed and non-pair fed groups, there were a few instances in which a mouse had to be separated from his cage mates due to his aggressive behaviors. When a mouse was isolated, it was offered an appropriate amount of chow for a single mouse, which usually consisted of one to three pellets at each feeding. Yet, we did not see a lack of weight gain in these individually housed mice that would support the theory of the lower weight of food in the hopper causing the mice to increase their energy expenditure and gain a lesser amount of weight. The only other possible explanation was that the pair fed mice experienced greater thermogenesis than did the LFU mice. We intend to explore this possibility when we analyze uncoupling protein-1 levels in the IBAT fat pads.

Mean weight gains were significantly greater when comparing LFU with LFO and LFU with PLU. Similar outcomes were observed in the studies published by Lopez-Varela, et al., and Garrido-Polonio, et al., when comparing rats fed unused oil with rats fed used oil (20, 21). This is interesting because the LFU and LFO mice in our study, as well as the animals in the previous studies, were all allowed to feed freely, were fed isocaloric diets, and their mean food intakes were not significantly different. Yet, in spite

of the isocaloric diets and food intake patterns, mean weight gains were different. This phenomenon might be explained by the difference in type of fat (unoxidized versus oxidized) that was incorporated into the diets. Interestingly, Chao, et al., conducted a study on the effects of thermally oxidized soybean oil in rats, with the oil heated repeatedly by means of frying wheat dough. This study found no difference in weight gains between the rats fed a low fat diet consisting of oxidized oil compared to the rats fed a low fat diet consisting of unoxidized oil (9). Perhaps this lack of difference could be explained by the overall very low percentage of fat in the low fat diets (5% of total calories was from fat) in Chao's study. For the current study and at the present time, we can only hypothesize that the Ox-LA fed mice gained less weight because the presence of Ox-LA in their diets caused them to lose skeletal muscle mass or increased their rates of thermogenesis in comparison with the Unox-LA fed mice. As stated previously, differences in thermogenesis may be examined as a possible explanation for the differences in weight gain when we quantify the levels of uncoupling protein-1 in the IBAT fat pads. Our study protocol did not include an analysis of lean body mass and as such we cannot comment on the possibility of lean muscle mass lost in the Ox-LA mice as being the reason for the lesser amount of weight gained by these mice.

In our study, diets were isocaloric and mean food intake was not significantly different when comparing any of the three low-fat fed groups. The same lack of significant difference in food intake was reported by Lopez-Varela, et al., and Garrido-Polonio, et al. (20, 21). In Chao's publication, rats fed heated oil consumed significantly more food than those rats fed fresh oil. In our study, the oil was heated without the addition of food, thereby ensuring we could attribute our observations to the products of

thermal oxidation of the oil alone. At the present time, the reasons for the differences seen in food intake when comparing our study, Lopez-Varela, et al.'s study, and Garrido-Polonio, et al.'s study with Chao's study remain inexplicable.

Feeding efficiency was calculated as a means to examine the relationship between total weight gain and total amount of food consumed. Mice in the LFU group were significantly more efficient in this respect than were the PLU mice. In other words, LFU mice gained significantly more weight per gram of food eaten than the PLU mice did. Although the LFO mice gained less weight per gram of food consumed than the LFU mice did, the difference was not significant. The same phenomena are observed in the studies by Lopez-Varela, et al. and Garrido-Polonio, et al. producing similar results to those of our study, while Chao's study showed no significant difference in feeding efficiency.

Mice in the LFO group had fat pads weighing significantly more than those of mice in the LFU and PLU groups, regardless of the type of fat pad. Significant differences were not seen in the fat pad weights of the LFU mice in comparison to the PLU mice. We find the greater fat pad weights of the mice fed Ox-LA to be evidence of the ability of dietary Ox-LA to regulate adipose mass accumulation in mice. No other study compared fat pad weights from as many different adipose tissue depots and, to our knowledge, ours is the first study to report that consumption of dietary oxidized lipids causes an increase in fat pad weights.

Our hypothesis proposed to answer the question of whether or not dietary oxidized lipids could lead to a change in adipose tissue *in vivo*; our results reveal the capacity of dietary oxidized linoleic acid to do so. With our knowledge of the capacity of

oxidized linoleic acid to be a ligand to PPAR γ , we proposed to examine whether or not the increase in adiposity seen was mediated by PPAR γ . Our analysis of PPAR γ protein levels in the cytosol of EWAT showed no significant differences between those mice fed unoxidized linoleic acid in comparison to those mice fed oxidized linoleic acid. At this time, PPAR γ protein levels in the nucleus of EWAT are still being analyzed. Because we have yet to complete our quantification of the amount of PPAR γ protein in all fat pads, as well as determine PPAR γ mRNA levels and activity levels of the protein in the tissues, we cannot make a statement at this time in regards to the role of PPAR γ in mediating the differences in weight gain and adiposity. In summary, it appears that the oxidation of linoleic acid is the reason for the lower food intake (although not significant), the significantly lesser amount of total body weight gained, and the significantly larger fat pads of those mice in the experimental group.

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