Diet-Induced Obesity in mice is associated with hyperinsulinemia, hepatic steatosis, and glomerulopathy in C57Bl/6J mice

Undi Hoffler^{1*}, Kristen Hobbie³, Ralph Wilson², Re Bai¹, Akef Rahman¹, David Malarkey², Greg Travlos², and Burhan I Ghanayem¹

^{1*}Laboratory of Pharmacology, and ²Cellular and Molecular Pathology Branch, National Institutes of Health, National Institute of Environmental Health Sciences, National Institutes of Health, and ³Integrated Laboratory Systems, RTP, NC

**To whom correspondence should be addressed*

Undi Hoffler

Laboratory of Pharmacology

National Institutes of Health

National Institute of Environmental Health Sciences

RTP, NC 27709

(919) 541-0427

Hoffler@niehs.nih.gov

Abstract

The prevalence and increasing incidences of obesity and obesity-related illnesses are not limited to westernized countries, but exist as a global epidemic impacting the health of adults and children. According to the WHO, over 1 billion adults are overweight ($\text{BMI} > 25 \text{ kg/m}^2$) and approximately one-third of this population has been diagnosed as clinically obese ($\text{BMI} > 30 \text{ kg/m}^2$). Causality between obesity and metabolic disorders (such as type 2 diabetes), cancer, cardiovascular diseases and liver diseases have been investigated using a variety of *in vitro* and *in vivo* models, including genetically engineered mice. The overall purpose of the current study was to fully characterize a genetically intact mouse of obesity for evidence of type II diabetes using clinical and anatomical pathology examination. Male C57Bl/6J mice were fed either a control diet or one in which 60% kcal were due to lard as early as 5 weeks of age and maintained on either diet through age 30 weeks. During the study, body weight and body fat measurements were obtained and complete necropsy was performed on mice at 15, 20, 30, and 40 weeks of age. Mice physiology was assessed and characterized through the analysis of serum chemistry (glucose, cholesterol, triglycerides, insulin, and leptin), histopathologic evaluation of tissues, and determination of hepatic and renal function. Significant increases in both body weight and body fat percentages were determined in male mice fed the high-fat diet vs. age-matched littermates consuming the control diet at 15, 20, 30, and 40 weeks. In concert with increased body weight and body fat %, serum cholesterol concentrations were significantly elevated throughout the study in obese vs. mice fed the control-diet. Serum insulin levels were ≥ 4 -fold higher in obese vs. control-diet fed mice by ages 30 and 40 weeks. 100% of male mice on the high fat diet

developed hepatic lipidosis (steatosis) by age 30 weeks with subsequent inflammation (steatohepatitis) by age 40 weeks. Obese male mice also exhibited mesangial cell and matrix proliferation (minimal to moderate) by 30 and 40 weeks of age. Notably, similar hepatic and renal lesions have been identified in humans with obesity-related illnesses, non-alcoholic fatty liver disease (NAFLD) and/or type II diabetes. The current genetically intact animal model may prove valuable in achieving the obesity phenotype in a manner appropriate to the onset of obesity and obesity-related diseases in humans.

Introduction

The global prevalence of obesity and obesity-related illnesses is increasing at staggering rates. Between 1985 and 2003, the number of diagnosed cases of obesity increased from 6.1% to 15.7% with the most severe forms of obesity (BMI > 35 kg/m²) exhibiting a 4.3-fold increase (Katzmarzyk and Janssen 2004). In parallel, there is a concomitant increase in obesity and hypercholesterolemia in very young children and adolescence (WHO 2006).

Obese individuals are susceptible to the development of type II diabetes, a metabolic disorder that is primarily characterized by insulin resistance and hyperglycemia. In addition to increased susceptibility to type II diabetes, obese individuals are more prone to cardiovascular diseases, dyslipidemia, sleep apnea, osteoarthritis, hepatic and renal diseases, and hypertension (McTiernan 2005; Muoio and Newgard 2006; Poirier 2006; Wolk and Somers 2006). It has also been suggested that obesity increases susceptibility to various cancers (Meyer 2008; Calle 2004; Bianchini 2002).

Studies have identified insulin resistance as a prerequisite biomarker to the onset of metabolic maladies associated with obesity (Reaven 2003; Wilcox 2005). Inflammation is another factor contributing to the pathogenesis of certain diseases in obese patients. Of particular interest are increased blood levels of inflammatory markers: C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α) (Bullo et al. 2003; McLaughlin et al. 2002; Roytblat et al. 2000; Yudkin et al. 1999). In addition to inflammation, obese and type II diabetic patients have also demonstrated increased activity of many hepatic metabolizing enzymes including, but not limited to,

cytochrome P450s (CYPs). Studies have shown more than any other CYP, CYP2E1 plays an important role in the positive feedback relationship between inflammation and reactive oxygen species. This relationship is further substantiated in the disease states, obesity and type II diabetes, where evidence of CYP2E1 induction at the transcriptional and translational levels has been presented (Dey and Cedarbaum 2007; Wang et al. 2003; Oesha et al. 1994).

Numerous *in vivo* models have been introduced in order to replicate obesity and type II diabetes. However, the majority of these animal models have achieved this phenotype through gene deletions or point mutations (Yoshinari et al. 2006; Klebanov et al. 2005; Rossmeisl 2003). A primary target of these mutations has been the leptin receptor gene. Evidence has shown that adipose tissue is not only fundamental in metabolism, but is also a vigorous organ within the endocrine system responsible for the secretion of adipokines, such as leptin (Whitehead et al. 2006; Cinti 2005; Kern et al. 2001; Yudkin et al. 1999; Dandona et al. 1998; Schwartz et al. 1997; Folsom et al. 1993). Leptin is an essential hormone involved in the regulation of body weight, metabolism, and reproductive function. In both animal models and humans, data has indicated that leptin deficiency does not automatically lead to the onset of diabetes or adiposity in humans. In the current paper, the research presented here was conducted using genetically intact C57Bl/6J male mice fed (ad libitum consumption) either a control (10% fat) or high-fat (60% fat) diet. This should more aptly mirror the developmental progression of obesity and type II diabetes in humans. Prior studies have utilized similar mouse models, but have lacked a complete description of the phenotype. Thus, the

current work will present a complete characterization of the model and thereby support its potential utility to further study obesity and obesity-related illnesses.

Materials and Methods

Animals:

Ten-week old male C57Bl/6J mice, fed a diet containing either 10% of the kcal from fat (control) or 60% (high fat), were purchased from Jackson Laboratories (Bar Harbor, ME). Both high fat and control diets, D12492i and D12450Bi respectively, were obtained from Research Diets, Inc (New Brunswick, NJ) provided to mice between the ages of 5 and 6 weeks old. C57Bl/6J mice were maintained ad libitum on either the control or high fat diets through 40 weeks of age at the National Institute of Environmental Health Sciences. All animals were housed individually in facilities with a 12-hr light-dark cycle with unfettered access to food and water. All animal care and experimentation were conducted according to NIH guidelines (US Department of Health and Human Services, 1986).

Body Fat Assessment:

Mice were weighed once a week and body fat measurements were determined at 15, 20 and 30 weeks of age. Body fat composition was acquired by dual-energy x-ray absorptiometry (DEXA) using the Piximus® densitometer (GE Medical Systems, Waukesha, WI). Mice were anesthetized using a mixture of 2.5-3% isoflurane during the analysis. This mixture enabled the quick recovery of each animal once removed from the densitometer.

Histopathology:

Complete necropsy was performed on subsets of C57Bl/6J mice at ages, 15, 20 and 30 weeks. Mice were sedated using CO₂ and blood was drawn from the retro-orbital venous sinus using heparinized capillary tubes. After blood was obtained, mice were

euthanized via CO₂ asphyxiation, tissues were collected immediately, fixed in 10% formalin for 18 hours, and then transferred to 70% ethanol for histopathological and immunohistochemical analysis. Histopathologic evaluation was performed on all male C57Bl/6J mice at ages 15, 20, 30, and 40 weeks. Following initial routine H&E evaluation, 3 µm sections of kidney were prepared and evaluated with Periodic- Acid Schiff (PAS). PAS staining of 3 µm kidney sections provided for a more prudent evaluation of the renal lesion. Using 3 µm kidney sections decreased the possibility that ‘depth of section’ influenced the identification of the lesion as glomerular mesangial cell hyperplasia. The following is a listing of all tissues examined via routine 5 µm H&E staining: brain, spinal cord, eye (with optic nerve, if possible), harderian glands, Zymbal’s glands, lacrimal glands, nasal cavity, pituitary gland, adrenal glands, thyroid gland, parathyroid glands, trachea, esophagus, salivary glands (submandibular, sublingual, and parotid), thymus, lungs, heart, aorta, spleen, kidney, liver, gallbladder, stomach, pancreas, duodenum, jejunum, ileum, cecum, colon, lymph nodes (mandibular and mesenteric), urinary bladder, skin, bone marrow, muscle, testes, epididymides, prostate gland, seminal vesicles, and preputial glands.

Grading of hepatic lipidosis was conducted using a 4 scale grading scheme in which increases of cytoplasmic fat were minimal if affected 1-25% of tissue, mild if 25-50% of tissue affected, moderate if 50-75% of tissue affected, and marked if 75-100% of tissue affected.

Glomerular mesangial proliferation severity was graded by percentage based on the number of glomeruli affected/ total number glomeruli for a given kidney section. The grades were as follows:

1. Grade 0 (Normal): Glomeruli had no increase in mesangial cells and mesangial matrix.
2. Grade 1+ (Minimal): Majority of glomeruli had a 1-25% increase in mesangial cells and mesangial matrix.
3. Grade 2+ (Mild): Majority percentage of glomeruli had a 25-50% increase in mesangial cell and mesangial matrix.
4. Grade 3+ (Moderate): Majority percentage of glomeruli had a 50-75% increase in mesangial cells and mesangial matrix.
5. Grade 4+ (Marked): Majority percentage of glomeruli had a 75-100% increase in mesangial cells and mesangial matrix.

Mesangial cell counts from PAS-stained kidney sections were also performed on 10 randomly selected glomeruli/mouse/diet at 30 and 40 weeks of age. Each glomerulus counted had a diameter of 100-150 microns. In addition to size, the presence of both vascular and urinary poles was favored.

Blood Chemistry:

Whole Blood collected from the retro-orbital venous sinus was captured in BD Microtainer® Serum Separator Tubes (Suwanee, GA) and centrifuged for the separate collection of serum. The following biochemical variables were assessed in all serum samples on an Olympus AU400e Clinical Chemistry Analyzer, Olympus America Inc (Irvin, TX). Using reagents obtained from the instrument manufacturer, serum glucose, triglycerides, cholesterol, and blood urea nitrogen (BUN) concentrations, and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities determined. Insulin concentrations were determined using two immunoassay methods, a radioimmunoassay (RIA) method using reagents obtained from Linco Research (St Charles, Missouri) on an Apex Gamma Counter, ICN Micromedic Systems Inc., (Huntsville, Al) and an electrochemiluminescent method using a Sector Imager 2400 and reagents from Meso Scale Discovery (MSD) (Gaithersburg, MD). Serum leptin

concentrations were generated simultaneously with the serum insulin concentrations using the MSD technique. Mouse specific reagents and procedures for insulin and leptin were obtained from the instrument manufacturer.

Glucose Tolerance Test:

Animals fed control or high fat diet for 30 weeks were fasted 16 hours with full access to water. Fasted animals were administered D-glucose solution at 2 g/kg body weight. D-glucose dosing solution was prepared in 0.9% saline and administered (ip). Untreated animals were vehicle (ip). At 30, 60 or 120 minutes after administration, mice were anesthetized with CO₂ asphyxiation and blood was drawn from the retro-orbital venous sinus. Serum was analyzed for the following: glucose, insulin, and leptin.

Statistical Analysis

All data presented are expressed as means $\pm$ standard error. Group mean comparisons were achieved using Microsoft Excel Student's *t* test and Trend Analysis was performed using SAS version 9.1. Repeated measures analysis of variance and Kruskal-Wallis analysis of variance, a non-parametric ANOVA method was used. Values were considered statistically significant at $P \leq 0.05$.

Results

Body Weight and Body Fat

A significant increase in body weight was observed in male mice consuming the high fat diet in comparison to age-matched littermates eating the control diet as early as 12 weeks of age and persisted throughout the study. C57Bl/6J mice on the high fat diet from 12-23 weeks of age experienced weight gain at a rate faster than mice of the same age consuming the control diet. This rapid rate of weight gain in high fat fed mice was also quicker during this earlier time than from 24-30 weeks of age. Strikingly, between 30 and 40 weeks of age, the rate of weight gain in both high fat and control diet fed mice escalated. At age 40 weeks, mice consuming a high fat diet averaged 60 grams, while age-matched littermates on the control diet weighed an average of 42 grams (Figure 1A). In addition to body weight, % body fat between the two groups was notably different. At all time points: 15, 20, 30, and 40 weeks, a significant increase in the body fat composition of mice on the high fat diet was seen in comparison to mice on the control diet, 29% vs. 18%, 36% vs. 21%, 36% vs. 26%, and 41% vs. 34% respectively (Figure 1B). In agreement with the pattern of weight gain observed in males fed a high fat diet, a plateau in their accumulation of body fat was observed at 20 weeks of age and similarly a significant rise was also observed between 30 and 40 weeks of age. In contrast, mice fed the control diet exhibited a deliberate increase in % body fat from ages 15-30 weeks. However, between ages 30-40 weeks a dramatic increase in the rate of amassing body fat was observed in mice fed the control diet vs. mice on the high fat diet.

Serum Biochemistry: Glucose, Insulin, Triglycerides ,and Cholesterol

High Fat vs. Control Diet Mice

Compared to age-matched controls mice consuming the high fat diet demonstrated statistical differences in various biochemical evaluations of carbohydrate and lipid metabolism at 15, 20, 30, and 40 weeks of age (Table 1). Overall glucose levels in males fed the high fat diet were higher at ages 15-30 weeks in comparison to mice of the same age fed the control diet. Statistically relevant differences in serum glucose, however, were seen at 20 and 30 weeks of age (Table 1, Figure 2). As mice approached 40 weeks old, serum glucose was remarkably similar, 190.3 ± 14.1 vs. 205.2 ± 11.0 mg/dl, in high fat and control diet, respectively (Table 1, Figure 2). An appreciable increase in serum cholesterol was determined in mice on the high fat diet at all ages in comparison to mice on the control diet (Table 1). Similarly, serum insulin was considerably higher in mice on the high fat vs. control diet at all ages, while statistically different at ages 30 and 40 weeks, >4-fold, respectively (Table 1, Figure 2). Triglyceride levels were also slightly elevated in high fat vs. control diet fed mice, but significantly different only at 40 weeks of age.

Mice on High Fat Diet

A measurable effect as a consequence of age and consumption of a high fat diet was determined in mice at 40 weeks old (Table 1). An approximate 20% decline in serum glucose was determined at 40 weeks of age in comparison to measured concentrations at age 15 weeks (Table 1, Figure 2) indicating a negative trend ($p=0.021$). In contrast, serum insulin levels remained progressive throughout the study demonstrating a positive trend ($p<0.0001$). By age 40 weeks, serum insulin

concentration increased approximately 5-fold in comparison to insulin levels determined in mice at 15 weeks of age (Table 1). Statistically important increases in serum cholesterol were also assessed at 20, 30, and 40 weeks in comparison to initial concentrations determined at 15 weeks of age (Table 1). By age 40 weeks, mice consuming a high fat diet presented with serum cholesterol concentrations that were approximately 68% higher than at age 15 weeks. These data, like serum insulin yielded a significantly positive trend ($p<0.0001$). Triglycerides were also significantly higher (1.6-fold) at 40 weeks age in comparison to age-matched mice on the control diet.

Mice on Control Diet

As control diet fed mice progressed in age changes in several biochemical parameters were evident. Through age 30 weeks, insulin levels remained relatively unchanged. However, at age 40 weeks an approximate 2-fold increase in serum insulin concentration (Table 1, Figure 2) was determined yielding a significantly positive trend ($p=0.006$). Slight fluctuations in the concentration of serum glucose were also demonstrated throughout the study (Table 1, Figure 2). A positive trend in serum cholesterol was also determined in mice consuming the control diet ($p=0.005$). By age 40 weeks, serum cholesterol had increased approximately 20% from initial values at 15 weeks of age. Conversely, serum triglycerides were significantly reduced in mice consuming the control diet by age 40 weeks. An estimated 25% decline in serum triglycerides from 15 to 40 weeks of age (Table 1) was established yielding a negative trend ($p=0.022$).

Blood Biochemistry: Fasted vs. Nonfasted

Distinctions in blood chemistry between fasted and nonfasted animals on either diet were determined using mice at 30 weeks old in order to alleviate the impact of age-related responses. MSD technique was used to support insulin results initially determined using RIA. Fasting significantly influenced the serum concentrations of both leptin and insulin in mice fed the control diet with differences approximating 2 and 3-4-fold, respectively (Table 2). Similarly, mice on the high fat diet exhibited 2-3-fold increases in the concentration of serum insulin when food was available (Table 2). However, in contrast to control mice, fasting did not affect serum leptin concentrations of mice on the high fat diet (Table 2). At 30 weeks of age serum leptin in fasted or nonfasted mice on a high fat diet was nearly identical (Table 2). Overwhelmingly, fasted mice on the high fat diet exhibited significantly higher concentrations of serum leptin and insulin in comparison to fasted mice on the control diet. Serum leptin and insulin were approximately 6 and 5-fold higher, respectively in fasted mice consuming the high fat vs. control diet. Interestingly, the serum concentration of insulin in fasted high fat mice was higher than serum insulin in control mice privy to food (Table 2).

Glucose Tolerance Test

At 30 weeks of age, male mice on the high fat diet had significantly elevated fasted glucose levels in comparison to age-matched males consuming the control diet, 199.5 ± 7.5 vs. 229.5 ± 9.1 mg/dl, respectively (Figure 3). Following glucose i.p. administration, animals on both diets experienced increased serum glucose levels at 30, 60, and 120 minutes. Clearance of serum glucose slowed in mice on the high fat vs. control diet between 60 and 120 minutes post dosing (Figure 3). The concentration of

glucose determined in the serum at 60 minutes, 338 ± 13.8 ng/ml was not statistically different from values obtained at 120 minutes, 293.2 ± 16.9 ng/ml (Figure 3). In contrast, mice on the control diet demonstrated a sustained and significant decline in serum glucose during that time (Figure 3). Higher concentrations of glucose persisted in mice fed the high fat vs. control diet and statistically important differences between groups were identified at 0 and 120 minutes post dosing (Figure 3).

Hepatic Function and Renal Enzyme Activity

At 30 weeks of age, blood urea nitrogen (BUN) as an indication of kidney function was similar in mice consuming the control or high fat diets, 21.7 ± 1.7 mg/dl and 23.8 ± 1.0 mg/dl, respectively. At age 40 weeks, mice on the control diet maintained BUN levels at 20.3 ± 1.1 mg/dl, while a significant increase in BUN was detected in mice on the high-fat diet, 26.3 ± 0.4 mg/dl (Table 3). Serum activity levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly higher in high fat fed 30 and 40-week old male mice vs. age-matched males on the control diet, approximately 3 and 2-fold, respectively (Table 3).

Histopathology

Liver

As early as 20 weeks of age, 50% (4/8) of male mice consuming the high fat diet developed hepatic lipidosis (steatosis) (Table 4). By age 30 weeks, the numbers of mice presenting with this lesion increased to 100% (8/8) and by age 40 weeks, a progression in the severity of the lesion was characterized by inflammation associated with steatosis (6/6) (Table 4). The lipidosis or steatosis was in the form of microvesicular vacuolation of hepatocyte cytoplasm and primarily identified in the centrilobular region (Figure 4B).

Microvesicular vacuolation was characterized by multiple, small, round, clear vacuoles within the cytoplasm of hepatocytes, which are consistent with accumulations of lipid (Figure 4B). Predominantly, the distribution of the lesion was centrilobular, but often expanded to include hepatocytes of mid-zonal regions (Figure 4B). The severity of the hepatic steatosis was graded according to percent of liver involved and generally increased in severity with time (Table 4). Lesion severity in mice consuming the high fat diet ranged from mild to marked at ages 30 to 40 weeks and only moderate in male mice at age 20 weeks. By 40 weeks of age, the lipidosis was accompanied by scattered infiltrates of mixed inflammatory cells (mononuclear and occasionally granulocytic) with rare degenerate hepatocytes, possibly an indication of early steatohepatitis (Figure 5). Also within the liver some hepatocytes contained large, round, clear vacuoles (macrovesicular vacuolation, primarily periportal and presumably lipid), sporadic occurrences of inflammatory infiltrate unrelated to steatosis, as well as lacy vacuolation (consistent with glycogen), but these findings did not appear to be diet or age-related.

Kidney

All (8/8) male mice on a high fat diet developed mesangial cell and matrix proliferation (minimal to moderate) by 30 weeks of age (Table 4, Figure 6). Glomerular mesangial cell hyperplasia was initially observed in the H&E-stained, 5 μ m kidney sections in 4 males at 30 weeks of age fed the high fat diet (and later confirmed in all 8 males using Periodic Acid Schiff [PAS]) (Table 4). PAS staining of 3 μ m kidney sections provided for a more prudent evaluation of the renal lesion. Using 3 μ m kidney sections decreased the possibility that ‘depth of section’ influenced the identification of the lesion as glomerular mesangial cell hyperplasia. This lesion was characterized by

variably increased numbers of mesangial cells with segmental diffuse expansion of the mesangium by pale, homogeneous, eosinophilic material and thickened glomerular basement membranes (Figure 6B). As the severity of the lesion increased, glomeruli would demonstrate a slight angularity to peripheral capillary loops.

Glomerular mesangial cell hyperplasia with increased mesangial matrix was found in (8/8) 30 week and (6/6) 40 week old mice on the high fat diet (Table 4). Glomeruli had approximately 30% increased numbers of mesangial cells with mesangium variably expanded by hypereosinophilic matrix (Figure 6,7). There were no notable changes within the glomeruli of any mouse at 30 weeks of age fed the control diet, however, at 40 weeks old, mesangial cell counts demonstrated an approximate 40% increase suggesting glomeruli were continuing to grow with age (Figure 6,7). Hyperplasia identified in (5/6) 40 week control mice was characterized as minimal to mild (Table 4). However, hyperplasia identified in (6/6) mice on the high fat diet at age 40 weeks was characterized as mild to moderate indicating a significant diet-related increase in the severity of the lesion as compared to mice fed the control diet through age 40 weeks, 2.7 vs. 1.2, respectively (Table 4).

Discussion

According to the World Health Organization (WHO), more than 1 million adults are considered overweight ($\text{BMI} > 25\text{kg/m}^2$) and approximately one-third of this population has been diagnosed as clinically obese ($\text{BMI} > 30\text{ kg/ m}^2$) (WHO 2005). Still, the global burden of obesity is projected to further escalate with 2.3 billion adults anticipated to be overweight by 2015 and 700 million of these individuals classified as obese. Numerous studies have suggested that obese individuals are at greater risk in developing an array of chronic illnesses including metabolic disorders such as type II diabetes and cancer. Although, *in vivo* models have been employed in order to study obesity and type II diabetes the development of these phenotypes have primarily resulted from a gene mutation or deletion. Whereas, the manifestation of obesity and type II diabetes in humans occurs foremost through the consumption of a diet high in fat and a sedentary lifestyle. Therefore, the purpose of the current research was to fully characterize a genetically-intact animal model that would approximate obesity and type II diabetes in humans.

Dietary composition played a significant role in the pathophysiology of obesity within the current study. Daily consumption of the high fat diet in which 60 kcal% were attributed to fat, primarily lard, imparted notable changes in male mouse blood biochemistry, histological alterations of the liver and kidney and altered hepatic function. Using a genetically-intact inbred mouse model, the presented work effectively replicated the onset of diet-induced obesity in humans as a chronic disorder characterized by a time-dependent and/or age-influenced progressive disease. As early as 12 weeks of age male

C57Bl/6J mice exhibited significant differences in weight in comparison to age-matched mice fed the control diet. By age 30 weeks, high-fat fed mice increased approximately 40% in body weight vs. mice on the control diet. Along with body weight, % body fat measurements were consistently higher in mice consuming the high fat diet. At 15, 20, 30, and 40 weeks of age body fat percentages in high fat fed vs. control diet fed mice, were 29% vs. 18%, 36% vs. 21%, 36% vs. 26%, and 41% vs. 34% respectively. Exacerbated by burgeoning weight and rising body fat percentages, circulating leptin levels at age 30 weeks in fasted animals on the high fat diet were markedly elevated, greater than 5-fold. Studies conducted in both humans and rodents have demonstrated a direct relationship between fasted serum leptin levels and body fat (Montague et al. 1997; Grinspoon et al. 1996). With diet as the dependent variable, current work supports earlier findings showing, as weight and body fat increased, sensitivity to leptin decreased fostering leptin resistance. Studies have proposed that adiposity inhibits the body's response to the regulatory actions of leptin through impaired leptin transport across the blood-brain barrier (Banks et al. 1999; Hileman et al. 2002; Banks et al. 2003; Wilsey et al. 2003; Banks et al. 2004). Banks et al. 2004 identified elevated triglycerides as the principal offender responsible for leptin's failure to penetrate into the CNS and bind to specific receptors located in the hypothalamus. Within the current work, in concert with hyperleptinemia, triglyceride levels in mice consuming the high fat diet were elevated in comparison to control diet fed mice at 40 weeks of age. An important extra-neuronal function of leptin is its direct involvement in the reduction of serum triglycerides (Steinberg et al. 2002). Success in this task is achieved by leptin through inhibiting free fatty acid synthesis, while promoting triglyceride hydrolysis and fatty acid oxidation

(Steinberg et al. 2002). Results within the presented work demonstrate over time a significantly negative trend in circulating triglycerides in mice fed the control diet, indicating these mice remained sensitive to the actions of leptin.

In the literature, differing hypotheses have been proffered regarding the influence of leptin on insulin secretion and vice versa (Schindler et al. 2006; Ceddia et al. 2002; Fehmann et al. 1997; Keiffer et al. 1997; Schwartz et al. 1997; Tanizawa et al. 1997). Collectively, studies have shown a continuous interplay between the two hormones; leptin acts as both an indirect and direct antagonist to insulin secretion and biosynthesis within the sympathetic nervous system and pancreatic β -cells, respectively (Seufert 2004). While the lipogenic actions of insulin has been shown to induce leptin biosynthesis and secretion from white adipose tissue (Seufert 2004). Within the current work there exists a positive correlation amongst adiposity and the ballooning levels of both leptin and insulin. The reduced efficacy of peripheral leptin correlates with the hypersecretion of insulin and presumed insulin resistance observed in our mice fed the high fat diet. It has been proposed that the inability of leptin to curtail glucose-stimulated insulin secretion from pancreatic β -cells in obese patients promotes chronic hyperinsulinemia and is a factor in the pathophysiology of type 2 diabetes (Seufert et al. 1999). In the current model, consumption of a high fat diet induced mild hyperglycemia as early as 20 weeks of age in concert with elevating plasma insulin. However, at 30 weeks of age a precipitous decline in glucose was evident and persisted through age 40 weeks. Serum glucose levels in 40 week old mice fed the high fat diet were comparable to lower glucose levels determined in mice fed the control diet at age 30 weeks. Despite this decrease in glucose, insulin levels continued to escalate in mice consuming the high

fat diet with concentrations at 30 weeks of age far exceeding age-matched controls (6-fold), serum insulin at 20 weeks of age in high fat mice (approximately 3-fold), and through age 40 weeks. In contrast, mice on the control diet at age 30 weeks exhibited significantly reduced levels (approximately 5-fold) of circulating leptin in parallel with consistently lower serum insulin and glucose concentrations. These data imply that the affect of hyperleptinemia on insulin secretion may be a more profound relationship than that shared between glucose and insulin in mice fed a high fat diet. In turn, the pathogenesis of obesity and obesity related illnesses within the current model may be predicated primarily in response to the dysregulation of the relationship between leptin and insulin rather than insulin and glucose.

Sustained exposure to hyperlipidemia in the blood and the accretion of fatty acids in non-adipose tissues as a consequence of obesity significantly amplifies the risk for the development of metabolic abnormalities and organ damage (Murray 2006; Nangaku et al. 2005; Lieber et al. 2004; Kambham et al. 2001; Garcia-Monzón et al. 2000). Studies have shown that an important purpose of leptin is to shield non-adipose tissues from retaining excessive fat stores through increased oxidation of fatty acids and decreased lipogenesis (Unger and Zhou 2001). In turn, the intimate relationship amongst adipose tissues, leptin, and fat stores suggest that leptin resistance is involved in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) and other progressive disorders including renal failure. Liver lipotoxicity resulting from excessive triglyceride deposition within hepatocytes and impaired fatty acid oxidation is a key contributor to the onset of insulin resistance and the development of NAFLD (Novartis Foundation 2008; Angulo 2002; Unger and Zhou 2001). Further, the potentiation of insulin resistance through

hyperinsulinemia and lipolysis encourages the accumulation of fat in liver cells (Angulo 2002). Within the current studies, moderate lesions, microvesicular vacuolation of hepatocyte cytoplasm, consistent with NAFLD were evident in 67% of mice fed the high fat diet at 20 weeks of age and at 30 weeks of age, mild to marked lesions were identified in 100% of mice evaluated. Although, the exact etiology of NAFLD has not been clearly elucidated in the literature, these data in support of earlier findings suggest that obesity characterized by hyperleptinemia and hyperinsulinemia in association with both leptin and insulin resistance presents as a significant risk in the development of NAFLD.

Interestingly, at 40 weeks of age mice on the high fat diet exhibited pronounced differences in liver histology in comparison to 30 week old mice consuming the same diet. Mononuclear inflammation juxtaposed with individual hepatocytes, biliary epithelial cell hyperplasia, and collagen deposition between liver cells were identified in livers from all mice on the high fat diet at age 40 weeks. These findings in addition to pronounced hyperinsulinemia and hyperleptinemia suggest a type II pre-diabetic state within these mice. Chronic-active inflammatory infiltrates and biliary hyperplasia associated with microvesicular vacuolation of hepatocytes suggest a response to hepatocellular injury, in particular, peroxidation of lipid accumulation within steatotic hepatocytes. In turn, this progression of pathology seen in 40 week old mice fed a high fat diet may represent an early progression of the hepatic steatotic lesion in steatohepatitis. In association with the gross pathology, liver dysfunction was apparent in this study based upon elevated levels of ALT and AST at 30 and 40 weeks of age in mice consuming the high fat diet. ALT and AST activity were increased approximately 3 and 2-fold, respectively in 30 and 40-week old obese mice in comparison to age-matched

control diet fed mice. To further correlate the presence of steatosis with attenuated liver function, sorbitol dehydrogenase (SDH), a non-inducible liver enzyme was also evaluated in 30-week old mice. SDH activity was greater than 2-fold higher in high fat vs. control diet fed mice indicating that the microvesicular vacuolation impaired the integrity of hepatocellular membranes causing leakage and dysfunction. It is our premise that dysregulation of hepatocellular function leads to increased susceptibility to environmental insult as a result of an inflammatory response and/or altered expression of metabolizing enzymes, particularly, cytochrome P450s (CYPs). Localization of the initial lesion, microvesicular vacuolation was prominent within the centrilobular region of the liver where CYPs, including CYP2E1 are most abundant. In the current study, at 30 and 40 weeks of age CYP2E1 expression was approximately 2-fold higher in high fat vs. control diet fed mice (data not shown). These data support earlier findings demonstrating that hepatic CYP2E1 is upregulated in animals and patients with non-alcoholic steatohepatitis (NASH) (Leclercq et al. 2000; Weltman et al. 1996; Weltman et al. 1998). CYP2E1 has also been shown to have a high inclination to generate reactive oxygen radicals and promote lipid and protein peroxidation in the presence or absence of substrates (Danko and Chaschin 2005; Caro and Cederbaum 2004; Lieber 1997). Studies investigating the role of CYP2E1 in the generation of oxidative stress within an animal model emblematic of NASH have shown a significant increase in the activity of microsomal NADPH-dependent lipid peroxidation in comparison to control animals (Leclercq et al 2000). Subsequent studies in hepatic microsomes from mice fed a methionine- and choline-deficient diet demonstrated 89% of NADPH-dependent lipid peroxidase activity was eliminated using an antiserum targeted specifically against

CYP2E1 (Leclercq et al 2000). In addition to the production of oxygen radicals via CYP2E1, another local source of oxidative stress within the current model can be directly attributed to increased levels of fatty acids and triglycerides sequestered within the hepatocytes of those mice consuming the high fat diet. The additive effect of CYP2E1-generated oxidative stress coupled with the innate oxidative actions of hepatocellular fatty acids may be responsible for the progression of hepatic lesions representative of NAFLD to steatohepatitis.

Obesity-related glomerulosclerosis has also emerged as a significant problem affecting the health of both adults and children (Kambham et al. 2001). Studies have shown a positive correlation amongst elevated serum leptin levels, hyperinsulinemia and the development of mesangial glomerulosclerosis (Cheol et al. 2000). Within the current study, a significant increase in the concentration of BUN in mice on the high fat diet was determined at 40 weeks of age in comparison to age-matched control diet fed mice. Further, glomerular mesangial cell hyperplasia was characterized in both 30 and 40-week old high fat mice as a significant increase in the number of mesangial cells and infiltration of hypereosinophilic material causing an expansion of the mesangium. However, the fate of these lesions is uncertain; they may be reversible and/or a precursor to glomerulosclerosis. Similar glomerular lesions, however, have been described in humans diagnosed with diabetic nephropathy. Patients with this diagnosis have exhibited mesangial cell proliferation, expansion of the mesangial matrix, and endothelial damage discernible as thickened glomerular basement membranes (GBM) (<http://www.merck.com/mmpe/index.html>). Human diabetic nephropathy has also been associated with numerous pathobiological events including: 1) insulin resistance and

hyperglycemia, which alter the GBM through increased collagen type IV and fibronectin synthesis, decreased heparin sulfate proteoglycan synthesis, nonenzymatic glycosylation of proteins and 2) increased glomerular filtration rate coinciding with increased glomerular capillary pressure resulting in glomerular hypertrophy and eventual renal failure (Kambham et al. 2001; Robbins and Cotran, 2006). Within the current model between 20, 30, and 40 weeks of age glucose levels declined significantly in mice consuming the high fat diet in association with inflated levels of both insulin and leptin. The development of these glomerular lesions in mice exhibiting hyperleptinemia and hyperinsulinemia lend further support to the suggestion that manipulations in the dynamic between leptin and insulin prompt a cascade of destructive biological occurrences in the kidney as well in other extrarenal tissues.

The identification of truncated isoforms of the predominant leptin receptor located in the hypothalamus, Ob-Rb, in murine mesangial cells suggests a direct mechanism for leptin activity within the kidney (Sweeney 2002; Lee et al. 1996; Tartaglia 1997). Leptin has also been shown to induce glomerular endothelial cell proliferation, stimulate type IV collagen synthesis and mRNA expression of transforming growth factor-beta1 (TGF- β 1). Studies have suggested that hyperleptinemia stimulates a paracrine TGF- β cross-talk between endothelial and mesangial cells of the kidney encouraging the development of glomerulosclerosis (Han et al. 2001). Furthermore, leptin has demonstrated independent mechanisms of inducing oxidative stress through the formation of reactive oxygen species (Bouloumié et al. 1999).

In conclusion, the current model is efficacious in achieving the obesity phenotype in a manner more appropriate to the onset of obesity and obesity-related diseases in

humans. Mice fed a high fat diet through 40 weeks of age demonstrated hyperinsulinemia and hyperleptinemia as well as elevated circulating triglycerides and cholesterol levels in comparison to mice fed a control diet. Although, mild hyperglycemia was initially evident in high fat fed mice, plasma glucose levels steadily declined over time indicating that the manifestation and progressive nature of lesions identified in these mice were most likely due to the dysregulation of the relationship between leptin and insulin. Proinflammatory actions of leptin in concert with insulin and leptin resistance are in need of further study, but are undoubtedly essential in the pathogenesis of metabolic disorders, obesity and type II diabetes, as well as may contribute to the onset of other obesity-related diseases.

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Table 1.

	<i>Age (weeks)</i>	<i>Glucose (mg/dl)</i>	<i>Insulin (ng/dl)</i>	<i>Triglycerides (mg/dl)</i>	<i>Cholesterol (mg/dl)</i>
Control Diet	15	184.0±9.0	1.7±0.3	117.3±14.1	162.2±4.2
	20	176.5±9.0	2.1±0.5 ^a	84.7±5.4	182.8±5.8 ^{†,α}
	30	193.3±8.0	2.3±0.3 ^β	96.6±6.3 ^β	177.9±8.7
	40	205.2±11.0	4.5±0.7 ^{a,†}	73.0±4.4 [†]	199.8±2.8 [†]
High Fat Diet	15	217.2±16.3	3.2±0.6	150.7±9.1	196.3±4.2 [×]
	20	242.2±16.2 ^{×,ω}	4.1±1.1 ^ω	103.3±8.3 [#]	225.0±9.0 ^{×,#,ω}
	30	213.6±4.9 [×]	10.5±1.2 ^{×,#}	103.5±6.7 [#]	281.4±8.9 ^{×,#}
	40	190.3±14.1	19.5±5.2 ^{a,×,#}	117.5±8.1 ^{×,#}	314.8±12.9 ^{×,#}

Legend: Mean ± SE. [×]Denotes a statistical difference in mice on the high fat diet vs. age-matched mice on the control diet ($P \leq 0.05$). ^aDenotes a statistical difference in mice age 40 weeks vs. mice 15, 20, and 30 weeks old ($P \leq 0.05$). Mice on control diet: [†]Denotes a statistical difference in mice on the control diet at 15 weeks of age vs. mice on the same diet at 20 and/or 40 weeks of age ($P \leq 0.05$). ^αDenotes a statistical difference in mice on the control diet at 20 weeks of age vs. mice on the same diet at 40 weeks of age ($P \leq 0.05$). ^βDenotes a statistical difference in mice on the control diet at 30 weeks of age vs. mice on the same diet at 40 weeks of age ($P \leq 0.05$). Mice on high fat diet: [#]Denotes a statistical difference in mice on the high fat diet at age 15 weeks vs. mice on the same diet at ages 20, 30, and/or 40 weeks ($P \leq 0.05$). ^ωDenotes a statistical difference in mice on the high fat diet at age 20 weeks vs. mice on the same diet at 30 and 40 weeks of age ($P \leq 0.05$). Serum used in the above analysis was collected from non-fasted mice. Reported serum insulin data was determined using radioimmunoassay. $N = 12, 12, 36$, and 12 at ages 15, 20, 30, and 40 weeks, respectively.

Table 2.

		<i>Leptin</i> (ng/ml)	<i>Insulin</i> (MSD) (ng/ml)	<i>Insulin</i> (RIA) (ng/ml)
<i>Control Diet</i>	<i>Fed</i>	12.6±1.9	4.0±0.6	2.3±0.3
	<i>Fasted</i>	6.6±1.0 [†]	0.9±0.1 [†]	1.0±0.3 [†]
<i>High Fat Diet</i>	<i>Fed</i>	34.8±2.8 [#]	16.2±3.2 [#]	10.5±1.2 [#]
	<i>Fasted</i>	32.6±1.8 [#]	4.5±1.2 ^{#,†}	5.4±1.3 ^{#,†}

Legend: Mean ± SE; [†] Denotes a statistical difference in fasted vs. fed mice on either the control or high fat diets at age 30 weeks ($P \leq 0.05$). [#] Denotes a statistical difference in fed or fasted mice on the high fat diet at age 30 weeks vs. fed or fasted age-matched mice on the control diet ($P \leq 0.05$).

Table 3.

		<i>ALT</i> (U/L)	<i>AST</i> (U/L)	<i>BUN</i> (mg/dl)
Control Diet	30 weeks old	51.1±11.2	83.4±9.9	21.7±0.9
	40 weeks old	144.2±26.0 [#]	165.7±18.1 [#]	20.3±1.1
High Fat Diet	30 weeks old	184.7±15.0 [†]	156.7±9.8 [†]	23.8±0.6
	40 weeks old	411.4±38.6 [†]	321.5±40.8 ^{†,#}	26.3±0.4 [†]

Legend: Mean ± SE; [†] Denotes a statistical difference in mice on the high fat diet at ages 30 and 40 weeks vs. age-matched mice on the control diet, ($P \leq 0.05$). [#] Denotes a statistical difference in mice at age 40 weeks vs. mice on the same diet at 30 weeks of age, ($P \leq 0.05$).

Table 4:

Lesions: Liver and Kidney								
<i>Number of animals evaluated/age/ diet</i>	<i>H15</i> 6	<i>C15</i> 6	<i>H20</i> 6	<i>C20</i> 6	<i>H30</i> 8	<i>C30</i> 8	<i>H40</i> 6	<i>C40</i> 6
Liver, microvesicular vacuolation	0	0	4(67%)	0	8(100%)	0	6(100%)	0
-Lesion Severity	0	0	3.0	0	3.3	0	4.0	0
<i>Inflammation associated with steatosis</i>	0	0	0	0	0	0	6(100%)	0
<i>Biliary hyperplasia</i>	0	0	0	0	0	0	6(100%)	0
Kidney, glomeruli mesangial cell hyperplasia	0	0	0	0	8 [†] (100%)	0	6(100%)	5(83%)
-Lesion Severity					2.4		2.7	1.2

Legend: C-Control diet; H-High fat; 15, 20, 30, 40-age of mice in weeks. Data are presented as the number and % of mice exhibiting lesions for the liver and/or the kidney based upon H&E analysis or [†]PAS staining and the severity of the lesion.

Figure Legends

Figure 1. A. Body weight of male C57Bl/6J mice ad libitum consumption of either control or high fat diets through age 40 weeks. A significant difference in weight was observed in mice fed the high fat diet at 12 weeks. Data points represent the mean $\pm$ SE of 24 mice per group. B. Body fat percentage of male C57Bl/6J mice ad libitum consumption of either control or high fat ad libitum diets through age 40 weeks. A significant difference in body fat composition was observed in mice fed the high fat diet at 15, 20, 30, and 40 weeks. Data points represent the mean $\pm$ SE of 24 mice per group.

Figure 2. Relationship between plasma glucose and insulin concentrations in male C57Bl/6J mice at 15, 20, 30 and 40 weeks of age. Significant differences in glucose levels were determined at 20 weeks old in high fat diet vs. control diet fed animals. Serum insulin concentrations were statistically significant in mice fed the high fat diet at ages 30 and 40 weeks vs. age-matched control diet fed mice. At 40 weeks of age insulin levels in both control and high fat mice were statistically relevant in comparison to concentrations at 15, 20, and 30 weeks of age.

Figure 3. Glucose Tolerance Test in male C57Bl/6J mice fed the control or high fat diet at age 30 weeks. Statistical differences were determined between control and high fat mice at 0 (fasted) and 120 minutes post (ip) administration of 2-D glucose. Significant differences in serum glucose were determined in mice on the control diet at 30, 60 and 120 minutes. Significant differences in serum glucose were determined in mice on the high fat diet at 30 and 60 minutes. Data points represent the mean $\pm$ SE of 34 mice.

Figure 4. A. Normal liver from a control diet 30 week-old male mouse. B. Microvesicular vacuolation of hepatocyte cytoplasm in high fat, 30 week male mouse characterized by clear, round, microvesicular vacuoles (arrow) and centrilobular distribution.

Figure 5. Livers from high fat diet 40 week old male mice with inflammation associated with steatosis. Photomicrographs demonstrating mononuclear cell inflammation (A) associated degeneration and necrosis of individual hepatocytes (arrow); biliary epithelial cell hyperplasia and (B); collagen deposition between hepatocytes (C)

Figure 6. A. Normal glomerulus from control diet 30 week male mice (H&E- left, PAS- right). Defined capillary loops and thin glomerular basement membrane (arrows). B. Mesangial cell hyperplasia with mesangial expansion in high fat diet 30 week male mice (H&E- left, PAS- right). Indicative of the hyperplasia and expansion were increased numbers of mesangial cells and expanded matrix (arrows).

Figure 7. Mean mesangial cell counts of glomeruli from mice fed either control or high fat diets. [#]Statistical differences were evident in both control and high fat mice at 30 vs. 40 weeks of age ($p<0.0001$) and ($p=0.003$), respectively. [†]Statistical differences were determined between control and high fat fed mice at 30 vs. 40 weeks old, ($p<0.0001$) and ($p=0.003$), respectively.

A.

B.

A.

B.

A.

B.

C.

A.

B.