INTRODUCTION

Obesity is the most common nutritional disorder in the developed world and has recently surpassed smoking as the number one risk factor for fatal disease in the United States [1]. As sedentary lifestyles and high fat diets become more prevalent, so too does the epidemic of obesity that has now transcended barriers of race, gender and age and spread into all sectors of the population. Obesity is a state characterized by an imbalance in energy metabolism resulting from excessive food intake and decreased energy expenditure. This syndrome has become prevalent in the United States today because of many genetic, environmental and psychological factors [2].

It is obvious that changes in lifestyle have made a contribution to the current levels of obesity, however the mechanism of food storage is something that has been present in humans for millions of years. In prehistoric times the efficient storage of energy as fat was favored by evolution and this fact plays a major role in the determination of body weight in humans today, with an estimated 50-90% inheritability for obesity. In the body, the obesity-avoidance and starvation-avoidance responses are regulated by the homeostasis of energy stores, which sends signals to the hypothalamus in the brain and these signals are interpreted in order to initiate the appropriate response. Although these mechanisms that suppress food intake and increase energy expenditure were designed keep a person's system in balance, it is clear that they are not strong enough to fight against the environmental and psychological factors that favor obesity [2]. The higher the rate of obesity becomes in our society, the more diseases emerge that have a direct correlation with an unhealthy accumulation of fat in the body. For example type II

diabetes, which was previously seen in mainly middle-aged women is now showing up in overweight children. With the incidence of obesity in the US calculated to approach 40% by the year 2025 the emergence of obesity-related diseases shows no sign of slowing [3].

One such disease that has become prevalent in the last few decades is a form of liver disease called non-alcoholic steatohepatitis (NASH). NASH is similar in histology and pathology to the alcoholic form of the disease seen in heavy drinkers; however as opposed to showing a strong correlation with alcoholism, it is associated with obesity, insulin resistance, type II diabetes, and dyslipidemia. The steatotic liver is both a precursor and a result of NASH and is the beginning point of future complications that can ultimately lead to liver cell death, cirrhosis of the liver and end stage liver disease. The characterization and diagnosis of NASH is still in its beginning stages. As of now additional symptoms associated with NASH other than steatosis include hepatocyte ballooning, lobular inflammation, and fibrosis through the activation of hepatic stellate cells (HSC's). These initial symptoms were linked with the more severe development of cirrhosis and are associated with the rising incidence of hepatocellular carcinoma [3]. The possibility of NASH being linked to these complications highlights the need for further study into the causes and results of this disease in order to better treat and prevent its occurrence.

 It was previously thought that NASH affected mainly middle age obese and diabetic women, however with the spread of these conditions to other sectors of the population it is now seen in men and children as well. There is a strong link between obesity and

NASH, however the true cause of this link remains undetermined. A steatotic liver is the main precursor to the development of NASH, but steatosis on its own does not necessarily guarantee that a person will develop the disease [4]. The pathogenesis of NASH remains unclear, yet its elucidation is a necessary step in the development of effective prevention and treatment protocols. One possible factor that is implicated in the transition from a steatotic liver to NASH is oxidative stress, which is defined as a disturbance in the prooxidant/antioxidant balance in favor of prooxidants, which can potentially lead to cellular damage [5]. Previous evidence demonstrated the presence of oxidative stress in steatotic human livers as well as those exhibiting NASH similar to that seen in alcoholic steatohepatitis, which shares many common features with NASH.

One of the possible sources of the oxidative stress that may lead to NASH are the cytochrome P450 (CYP) enzymes present in the body. The human genome encodes fifty-seven CYP enzymes, fifteen of which are involved in the metabolism of drugs and xenobiotics. These enzymes were first recognized in the oxidation of drugs, carcinogens and steroids. The action of these enzymes follows the general oxidative scheme:

$$NADPH + H^+ + R + O_2 \rightarrow NADP^+ + H_2O + ROH$$
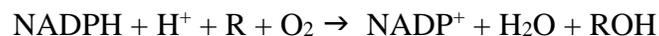
$$(R= substrate, ROH= product)$$

In mammals the majority of the CYPs are found in the endoplasmic reticulum where they interact with and receive electrons from the flavoprotein NADPH-P450 reductase. CYPs are also found in the mitochondria of the cell where they operate via an electron transport chain and utilize the flavoprotein adrenodoxin reductase.

Although the majority of CYP proteins function in the metabolism of endogenous substances such as steroids and vitamins, the main concentration of research over the past few decades has been on those CYPs that function in xenobiotic metabolism. Through this research it was found that of the 57 CYPs present in the body, only 5 are responsible for approximately 95% of the drug metabolism that takes place. Due to high expression levels and broad substrate selectivity, these CYPs are a major consideration for drug trials and a useful tool when predicting potential drug-drug interactions. Of the five major CYPs involved in drug metabolism, CYP2E1 was identified as being induced by ethanol in the liver, and is the main CYP examined when studying alcohol related liver disease [6].

In the case of alcoholic steatohepatitis, the presence of ethanol induces the concentration of CYP2E1. This CYP oxidizes ethanol and contributes to its metabolism. It was also documented that this process generates reactive oxygen species (ROS) in the body through the NADPH dependent reduction of O_2. ROS, such as the superoxide anion radical and hydrogen peroxide, cause damage to cellular membranes by initiating the process of oxidation of fatty acids in membrane lipids, a process called lipid peroxidation. This type of cell damage can lead to necrosis and apoptosis of liver cells and can contribute to alcohol-induced liver disease [5]. The increase of CYP2E1 in chronic ethanol exposure is well documented in the literature. Similar increases of CYP2E1 are observed in the metabolic states of diabetes, obesity and dyslipidemia as well [4,7-9]. These states are also commonly associated with NASH. This connection establishes a strong causal relationship between the oxidative stress caused by CYP2E1

and the development of NASH, which has prompted further investigation of the link

between increased CYP2E1 and its possible role in the production of oxidative stress.

One proposed pathway that would initiate lipid peroxidation as a result of increased

CYP2E1 in these metabolic states focuses on the enzyme's ability to hydroxylate free

fatty acids. In the liver, free fatty acids undergo β-oxidation or re-esterification to

triglycerides, which are then packaged into very low-density lipoproteins (VLDL's).

When the ability of the liver to make and secrete VLDL's is exceeded, triglycerides

accumulate resulting in steatosis [9,10]. As stated above steatosis alone is benign;

however, when it is accompanied by oxidative stress it can lead to the development of

NASH. Since CYP2E1 is known to hydroxylate fatty acids it is possible that when

hydroxylating these substrates there is an increase in the uncoupled reaction, thus

producing more reactive oxygen species and increasing microsomal lipid peroxidation.

From previous mouse studies there is in vitro evidence of an increase in microsomal lipid

peroxidation in association with NASH. It was shown that CYP2E1 is directly

responsible for this increase as evidenced by the reduction of lipid peroxidation when

CYP2E1 inhibitors were added. These conclusions led researchers to test the relationship

of CYP2E1 activity and the development of NASH in mouse models by looking at

Cyp2e1 -/- mice that lacked the ability to express this enzyme. It was expected that these

mice would not develop the histological conditions normally associated with NASH,

however these mice also exhibited a large increase in lipid peroxidation. This suggested

that there must be alternative pathways acting to produce oxidative stress in the

Cyp2e1 -/- mice. Immunoblot analysis and antibody inhibition studies suggested that CYP4A enzymes might be the source of the reactive oxygen species [4]. As a result, it is necessary to study CYP4A as well when investigating the role of CYP enzymes in the development of NASH.

As stated above, models employed in studying the role of the CYP enzymes in the development of NASH include mouse and rat models in response to conditions such as obesity and insulin resistance. Due to the similarities between the human and mouse genome in the CYP regions and the ability to easily manipulate their genes, mice present a model in which to study the progression of NASH and are applicable to the human form of the disease [11]. In a previous study from Leclercq *et al.* [4], mice were fed a methionine and choline deficient diet in order to interfere with the liver's ability to remove excess fat, thereby inducing steatosis. Other studies utilized genetic knock-out (ob/ob) mice that lack leptin and are therefore unable to regulate food consumption and fat metabolism, resulting in obesity [4]. This project utilized brain derived neurotrophic factor (BDNF) knock-out mice that develop mature onset obesity. BDNF is a neurotrophic factor that plays a role in regulating food balance and energy expenditure. Using the cre-loxP recombination system, conditional BDNF mutant mice were generated that expressed age related obesity characterized by an 80-150% increase in body weight, and hyperinsulinemia [12]. This makes them an excellent model to study this disease and attempt to discover any common factors that could be responsible for the progress of steatosis to NASH.

In this study the levels of CYP2E1 and CYP4A were studied in the BDNF knock-out mice in comparison with wild type mice in order to examine the relationship between their metabolic conditions and the levels of these enzymes. Once these levels were established, catalytic assays were performed to determine the activities of the enzymes that were present and ascertain whether there was an increase of CYP2E1 and CYP4A activity in the mice that also expressed obesity and hyperinsulinemia. Following the establishment of these relationships, an assay to measure lipid peroxidation was performed on the knock-out and wild type samples in order to establish a link between the levels of the CYP enzymes and the amounts of lipid peroxidation. The results of these experiments do not discount the hypothesis that CYP2E1 and CYP4A may be involved in producing the oxidative stress required for progression from steatosis to NASH.

METHODS

Animals. The animals used in this experiment were provided by Dr. Maribel Rios from

Tufts University School of Medicine (Boston, Massachusetts, USA). Using the cre-loxP

recombination system, two types of mutants were generated as outlined by Rios *et. al.*

[12]. The CamK-cre mice were brain BDNF knock-outs and expressed age related

obesity and hyperinsulinemia. The Alb-cre mice were liver BDNF knock-outs and did not

express the age related obesity associated with the brain knock-outs. All the animals in

this study were of mixed genetic backgrounds. Both the wild type and mutant mice were

fed a standard chow diet and water *ad libitum* [12]. Wild type mice that contained both

brain and liver BDNF genes as well as young knock-out mice that did not exhibit obesity

acted as controls for this experiment. The mice were sacrificed and their livers were

extracted to yield the following samples:

Table 1. Mouse Sample Information

Genotype	Gender	Age	Metabolic State
WT	F	10 weeks	lean
WT	M	9 weeks	lean
CamK-cre	M	10 weeks	lean
CamK-cre	F	8 weeks	lean
WT	F	7 months	lean
WT	F	7 months	lean
WT	F	7 months	lean
CamK-cre	F	7 months	obese
CamK-cre	F	7 months	obese
CamK-cre	F	7 months	obese
Alb-cre	M	7.5 months	lean
Alb-cre	M	7.5 months	lean
Alb-cre	F	7.5 months	lean
Alb-cre	F	7.5 months	lean

Microsome Preparation. Using the livers from these mice, microsome samples were prepared using differential centrifugation. Liver samples were weighed and homogenized in 4 volumes of cold homogenizing buffer (10 mM Tris, 0.25 M sucrose, 1 mM EDTA, pH 7.4). Homogenized tissues were transferred to microcentrifuge tubes and were centrifuged for 20 minutes at 10,000 x g at 4°C. The supernatant was removed and transferred to Beckman Tabletop centrifuge tubes. The tubes were centrifuged for 60 minutes in the TLA45 100.4 centrifuge at 100,000 x g at 4°C. The supernatant was removed and the pellet was resuspened in microsome storage buffer (10 mM Tris, 20% glycerol, pH 7.4) (w/v 1:2) using a motorized pellet pestle. The samples were transferred to microfuge tubes for protein concentration determination.

Protein Concentration Determination. The protein concentration of microsome samples was determined using a Bradford assay (Bio-Rad Protein Dye Reagent; Bio-Rad). The liver samples were diluted 1:100 in water. Bovine serum albumin (BSA) was diluted with water to make five standards ranging from 0 to 0.500 µg/µl. Five microliters of each standard as well as 5µl of the microsome sample dilutions were added in triplicate to the wells of a microplate. Two hundred microliters of 20% Bradford solution was added to each well and the absorbances of the samples were measured in the microplate reader at a wavelength of 595 nm. The BSA standards were used to generate a standard curve from which the concentrations of the microsome samples were extrapolated.

Determination of Microsomal CYP Proteins. The presence of microsomal CYP proteins was determined using western immunoblot analysis. Using the prepared microsome

samples, 100 ug aliquots were made by diluting to 1 $\mu g/\mu l$ with sample diluting buffer

(12.5 ml stacking buffer, 2.2% SDS, 0.1% pyronin y and bromophenol blue, 22.2%

glycerol, and 35.5 ml water) mixed with 5% by volume β-mercaptoethanol. Samples

were boiled for 1 minute and stored at -20°C between uses. The samples were run on an

SDS-PAGE gel and transferred to a nitrocellulose membrane. The CYP4A1 blot was

blocked in 5% non-fat dry milk overnight at 4°C. The remaining blots were blocked in

2.5% gelatin for 30 minutes at room temperature. The membranes were then incubated

using polyclonal goat anti-rat CYP2E1, rabbit anti-sheep CYP4A1, rabbit anti-rat

CYP3A2, or rabbit anti-rat CYP2C11 as the primary antibodies. Secondary antibodies

were anti-sheep, anti-goat or anti-rabbit horseradish peroxidase conjugated antibodies as

appropriate. Proteins were viewed via a chemiluminescent reaction using the Pierce

Supersignal West Dura Extended Duration Substrate (Pierce Biotechnology, Inc.,

Rockford, IL). Following exposure the images were captured by the UVP cooled CCD

system EpiChemi 2 or 3 darkroom and quantified using the Labworks software 4.0

(Labworks LIMS, Shelton, CT).

Determination of Enzymatic Activity. In order to determine CYP2E1 activity in the

microsome samples a chlorozoxazone-6-hydroxylase assay was performed. A 1.0 ml

reaction mixture containing 0.1 M Hepes (pH 7.6), 10x Regeneration System (5 mM

$NADP^+$, 100 mM glucose-6-phosphate, 100 mM $MgCl_2$), 10 mM chlorozoxazone, H_2O,

200 μg microsomes and 1 U/μl glucose-6-phosphate dehydrogenase was incubated for 10

minutes at 37°C. Following incubation the reaction was quenched with 50 μl of 43%

H_3PO_4. The samples were extracted twice with 3 ml ethyl acetate, vortexed for 15 seconds and centrifuged for 5 minutes at 1000 rpm. The top ethyl acetate layer was removed and evaporated to dryness under pressure. The samples were resuspended in 200 μl start solvent (83% Solvent A: H_2O with 0.25% acetic acid, and 17% Solvent B: 100% HPLC grade acetonitrile), vortexed and centrifuged in the tabletop centrifuge. The samples were transferred to autoinjector vials and were run on the HPLC using a Supelco Supelcosil LC-18 column (150 mm x 4.6 mm x 5 μm) and 7-OH-Coumarin as an internal standard. The retention time for 6-hydroxychlorozoxazone was approximately 6.0 minutes. The product was detected by monitoring the effluent at 296 nm and determining the peak area ratio of the metabolite and internal standard in comparison with standard curves generated from known amounts of product.

A p-nitrophenol (PNP) hydroxylation assay was also performed on the microsome samples. A 10 ml reaction mixture containing a master solution (2 mM KPi pH 6.8, 0.2 mM PNP and 2 mM sodium ascorbate), H_2O, and 200 μg microsomes was incubated for 3 minutes at 37°C. Ten microliters of 0.1 M glucose-6-dehydrogenase was added to each sample and the reaction was allowed to proceed for 5 minutes. The reaction was stopped with 0.2 ml perchloric acid and the samples were left on ice for 5 minutes. The samples were transferred to microfuge tubes and spun at 10,000 rpm in the microfuge for 5 minutes in the cold room. Following the spin, the samples were transferred to glass tubes and 0.1 ml of 10 N NaOH was added to each tube. Two hundred microliters from each tube was transferred in triplicate to a 96 well plate and the absorbance read at 510 nm.

The levels of reductase were also measured in the samples using the method of western immunoblot analysis as described above. The blot was blocked overnight at 4°C in 5% non-fat dry milk. The membrane was incubated in rabbit anti-pig reductase as a primary antibody. Goat anti-rabbit horseradish peroxidase conjugated antibody was used as the secondary antibody. The blot was developed and the results were quantified as previously described.

Determination of Lipid Peroxidation. The levels of lipid peroxidation for the wild type and BDNF brain knock-out samples were measured using the Oxis LPO-586 colorimetric assay kit (BIOXYTECH, Portland, OR). A 0.5 ml reaction mixture containing 0.1 M HEPES, 0.2 mg microsomes, 10x regeneration system (5 mM $NADP^+$, 100 mM glucose-6-phosphate, 100 mM $MgCl_2$), H_2O and 1 U/μl glucose-6-phosphate dehydrogenase was incubated for 60 minutes at 37°C. Following incubation, 200 μl were taken from each tube placed in a microfuge tube along with 650 μl of reagent 1 (N-methyl-2-phenylindole in acetonitrile) and 150 μl reagent 2 (methanesulfonic acid), which was incubated for 60 minutes at 45°C. The samples were centrifuged at 4°C and 15,000 x g for 10 minutes. Samples were transferred to a 96 well plate and their absorbance was read at 596 nm. Using the malondialdehyde (MDA) standards provided in the kit, a standard curve was generated and the amount of lipid peroxidation in each sample was extrapolated and quantified.

RESULTS

Effect of Obesity on CYP Protein Expression. Using the western immunoblot and quantification procedures described in the previous section the relative amounts of CYP2E1 and CYP4A were determined and the results were plotted in order to visualize the relative differences between genotypes (Figures 1 and 2). The levels of CYP2C and CYP3A were also measured and the results were plotted as a control in order to determine the specificity of enzymatic regulation (Figure 3 and 4). The levels of CYP2E1 in the BDNF brain knock-out mice were not significantly elevated in comparison with their wild type counterparts. The CYP4A levels in the brain knock-outs did exhibit a trend for elevation in comparison with the wild type mice; however, there was a high degree of variability within the samples, causing the average values to have a large standard deviation. Statistical analyses were performed and the results from these tests indicated that the differences in the CYP4A levels between wild type and brain knock-out were not statistically significant.

It was also seen from the graphs that the levels of CYP2E1 were elevated in the aged female liver knock-out samples with respect to the wild type mice and the aged male liver knock-out samples. Although this difference was not statistically significant (P = 0.0093) it was a result that was consistent through all runs, and was not seen in the control runs with CYP2C.

Effects of Obesity on Enzymatic Activity Levels The cholorozoxazone-6-hydrolysis assay was performed in order to measure the levels of enzymatic activity in the CYP2E1

proteins present in the microsome samples. From a plot of the nmol of 6-hydroxy-chlorozoxazone produced per mg protein present (Figure 5) it was seen that the aged brain knock-out samples did not exhibit elevated activity in comparison with the wild type samples. The results of this assay were consistent with the western immunoblot data in that the levels of CYP2E1 activity were elevated in the female liver knock-outs in comparison with their male counterparts.

The results of the PNP hydroxylation assay were also plotted in units of nmol of product produced per minute per mg of protein present (Figure 6). In contrast to the CZ assay, this measurement of CYP2E1 activity showed that there was elevation in the obese samples in comparison with the wild type samples, but with a high degree of variability. The aged female liver knock-outs expressed elevated activity in comparison with their male and wild type counterparts, which was consistent with the results of the CZ assay.

The levels of reductase present in the microsome samples were also measured as an indicator of the level of the enzyme that can be limiting for the enzymatic activity of the microsome samples. These levels were measured using western immunoblot analysis and a rabbit anti-pig reductase probe. The results from this blot were plotted using the same method as that for the previous immunoblot data (Figure 7). The results from this analysis indicated patterns that had been established through the previous tests with the levels of reductase not showing elevation in the brain knock-outs, but expressing elevated levels in the aged female liver knock-outs.

Effects of Obesity on Lipid Peroxidation Levels Finally an assay to measure the levels of lipid peroxidation in the brain knock-outs in comparison with the wild type samples was performed (Figure 8). The brain knock-outs that expressed obesity exhibited elevated levels of lipid peroxidation, however not enough to be statistically significant according to the t-test statistical analysis with a p-value of 0.299. The samples within each group exhibited a high degree of variability, which resulted in a large standard deviation. The levels of lipid peroxidation of the other microsome samples were not measured.
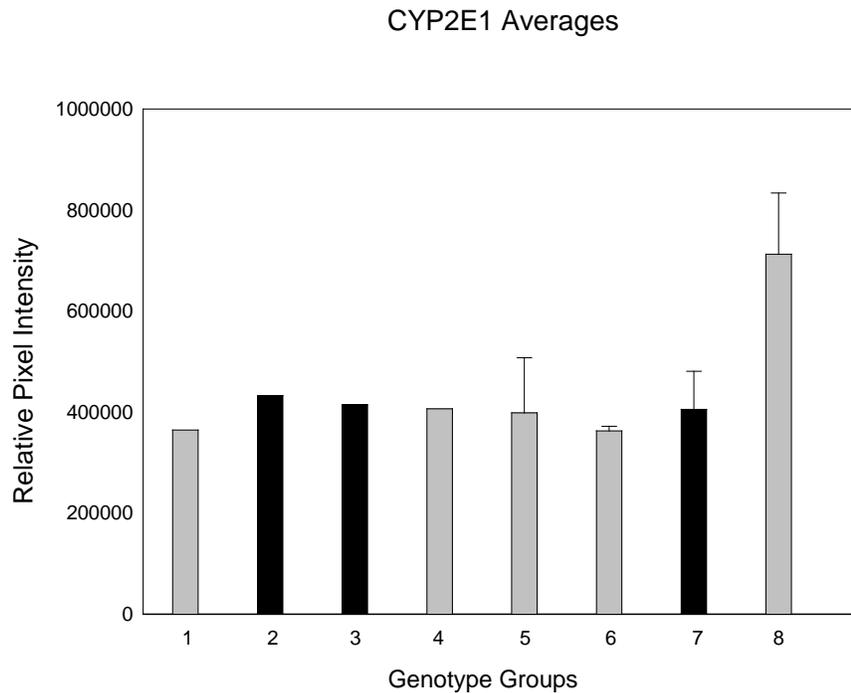
CYP2E1 Averages

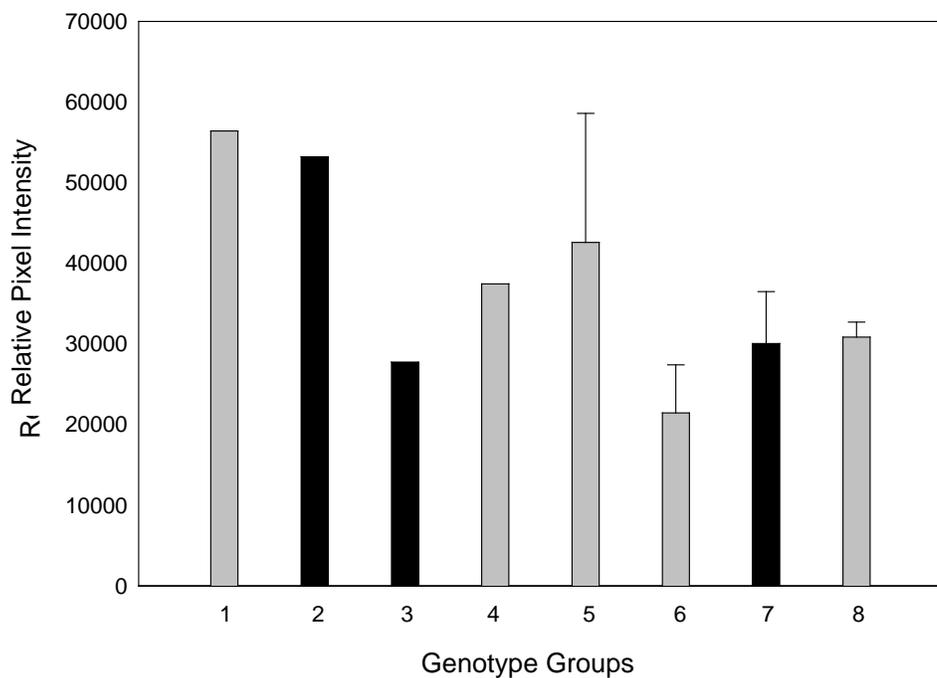| Bar #	Genotype (Gender)
1	WT- young (F)
2	WT-young (M)
3	Brain KO-young (M)
4	Brain KO-young (F)
5	WT-aged (F)
6	Brain KO-aged (F)
7	Liver KO-aged (M)
8	Liver KO-aged (F)

Figure 1. Average CYP2E1 expression levels in microsome samples. The obese BDNF brain knock-outs (6) did not express elevated levels of CYP2E1 in comparison with the wild type samples (5). The female liver knock-outs (8) exhibited elevated levels in comparison with their male counterparts, however this difference was not statistically significant.
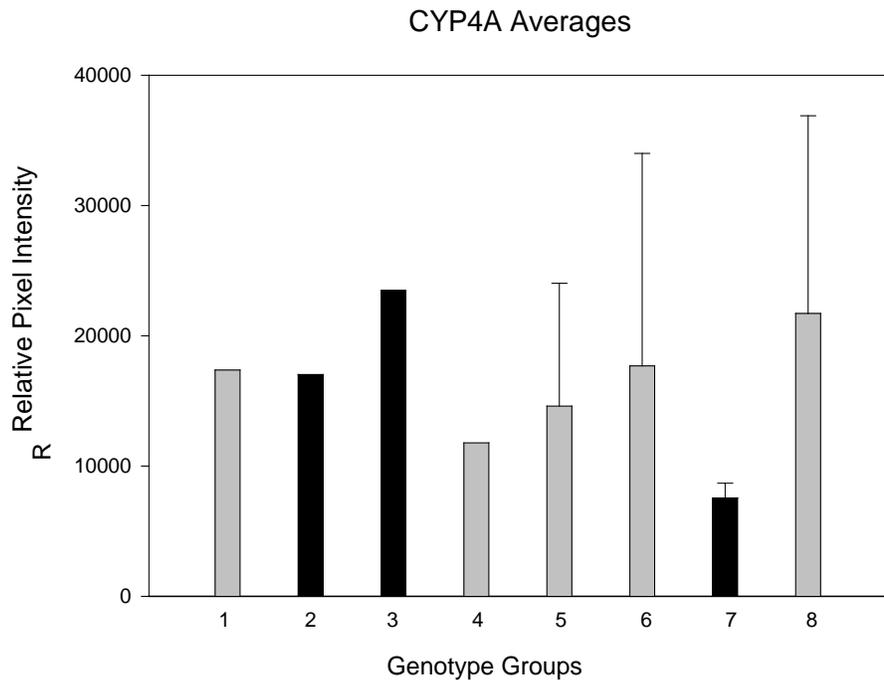
CYP4A Averages

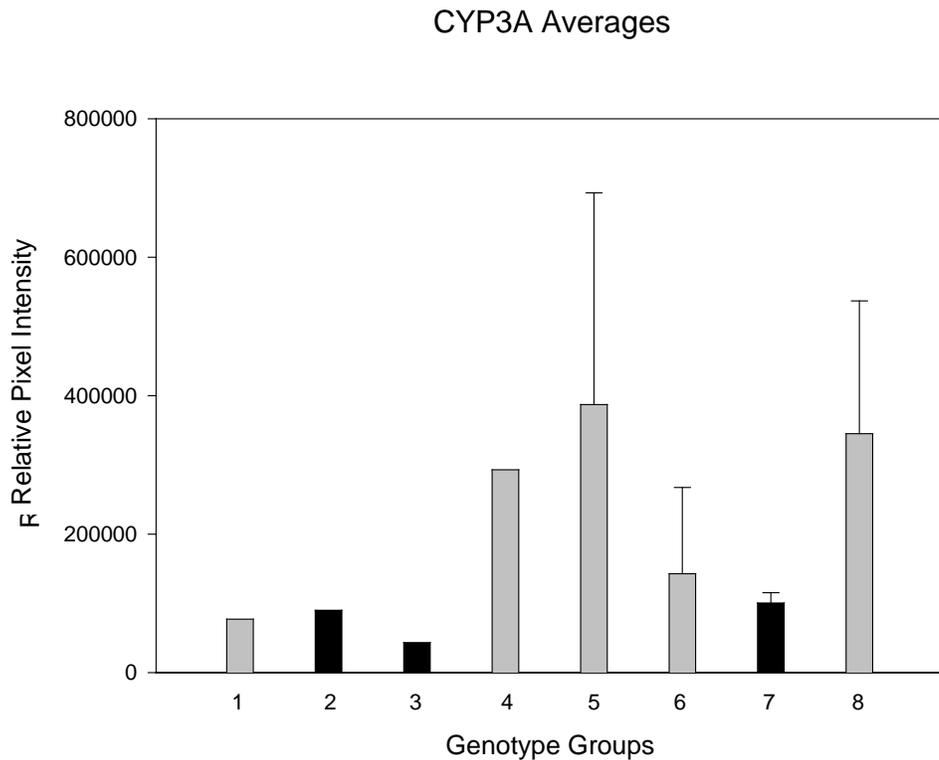| Bar #	Genotype (Gender)
1	WT- young (F)
2	WT-young (M)
3	Brain KO-young (M)
4	Brain KO-young (F)
5	WT-aged (F)
6	Brain KO-aged (F)
7	Liver KO-aged (M)
8	Liver KO-aged (F)

Figure 2. *Average CYP4A expression levels in microsome samples*. The BDNF brain knock-outs (6) exhibited a slight elevation in enzymatic expression, however from the large error bar it is apparent that there was significant variation among the samples.

CYP2C Averages

Bar #	Genotype (Gender)
1	WT- young (F)
2	WT-young (M)
3	Brain KO-young (M)
4	Brain KO-young (F)
5	WT-aged (F)
6	Brain KO-aged (F)
7	Liver KO-aged (M)
8	Liver KO-aged (F)

Figure 3. Average CYP2C expression levels in microsome samples. The CYP2C enzymes were measured as a control for this series of experiments. The levels of CYP's are not inherently high for the obese brain knock-outs (6) in comparison with the wild type mice (5). The male (7) and female (8) liver knock-outs also show similar levels of expression.
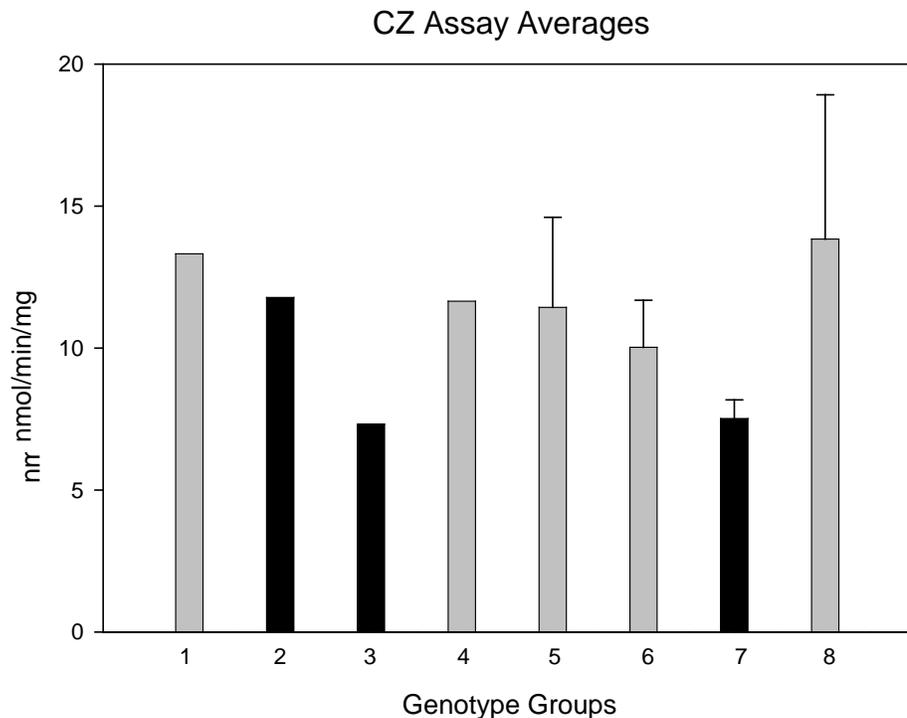
CYP3A Averages

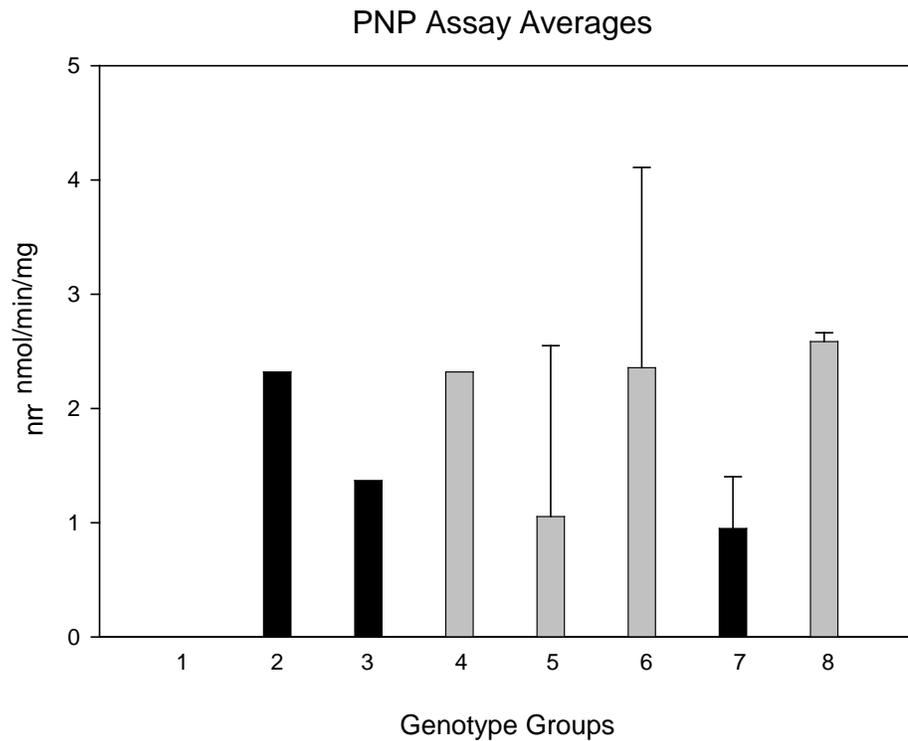| Bar #	Genotype (Gender)
1	WT- young (F)
2	WT-young (M)
3	Brain KO-young (M)
4	Brain KO-young (F)
5	WT-aged (F)
6	Brain KO-aged (F)
7	Liver KO-aged (M)
8	Liver KO-aged (F)

Figure 4. Average CYP3A expression levels in microsome samples. The levels of CYP3A were also measured as a control for these experiments. There is significant variation within the sample groups, which is a characteristic that made interpretation of the data difficult.

CZ Assay Averages

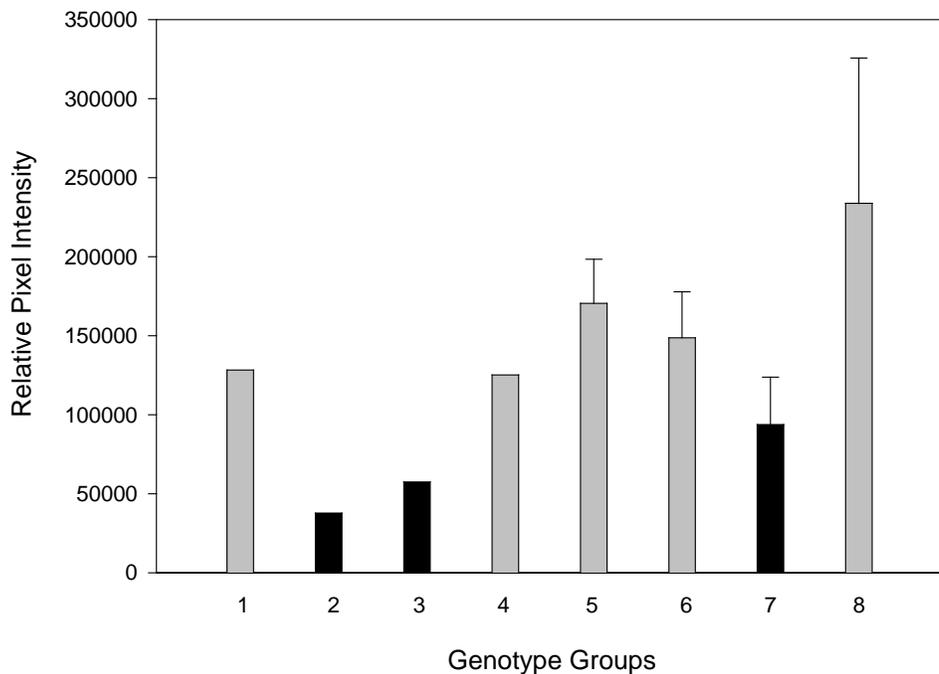| Bar #	Genotype (Gender)
1	WT- young (F)
2	WT-young (M)
3	Brain KO-young (M)
4	Brain KO-young (F)
5	WT-aged (F)
6	Brain KO-aged (F)
7	Liver KO-aged (M)
8	Liver KO-aged (F)

Figure 5. *Average CYP2E1 activity in microsome samples from the CZ assay.*
The CZ assay was performed in order to measure CYP2E1 enzymatic activity.
The results from this assay were consistent with the Western blot data and showed
that the activity levels were not elevated in the brain knock-out (6) versus the wild
type mice (5). The female liver knock-outs (8) again showed a slight elevation.

PNP Assay Averages

| Bar #	Genotype (Gender)
1	WT- young (F)
2	WT-young (M)
3	Brain KO-young (M)
4	Brain KO-young (F)
5	WT-aged (F)
6	Brain KO-aged (F)
7	Liver KO-aged (M)
8	Liver KO-aged (F)

Figure 6. Average CYP2E1 activity levels in microsome samples from the PNP assay. The PNP assay was performed to measure the levels of CYP2E1 enzymatic activity. In contrast to the CZ assay, this experiment showed that the levels of CYP2E1 activity were elevated in the obese samples, however there was a high degree of variability among them.

Rbt anti-pig Reductase Averages

Bar #	Genotype (Gender)
1	WT- young (F)
2	WT-young (M)
3	Brain KO-young (M)
4	Brain KO-young (F)
5	WT-aged (F)
6	Brain KO-aged (F)
7	Liver KO-aged (M)
8	Liver KO-aged (F)

Figure 7. Average levels of reductase expression in the microsome samples. The reductase levels were measured as an indicator of the level of the enzyme that can be limiting for the enzymatic activity of the microsome samples. The level of reductase seen in the obese knock-out samples (6) did not express elevation in comparison with the wild type samples (5), however the female liver knock-outs (8) did show signs of elevation.
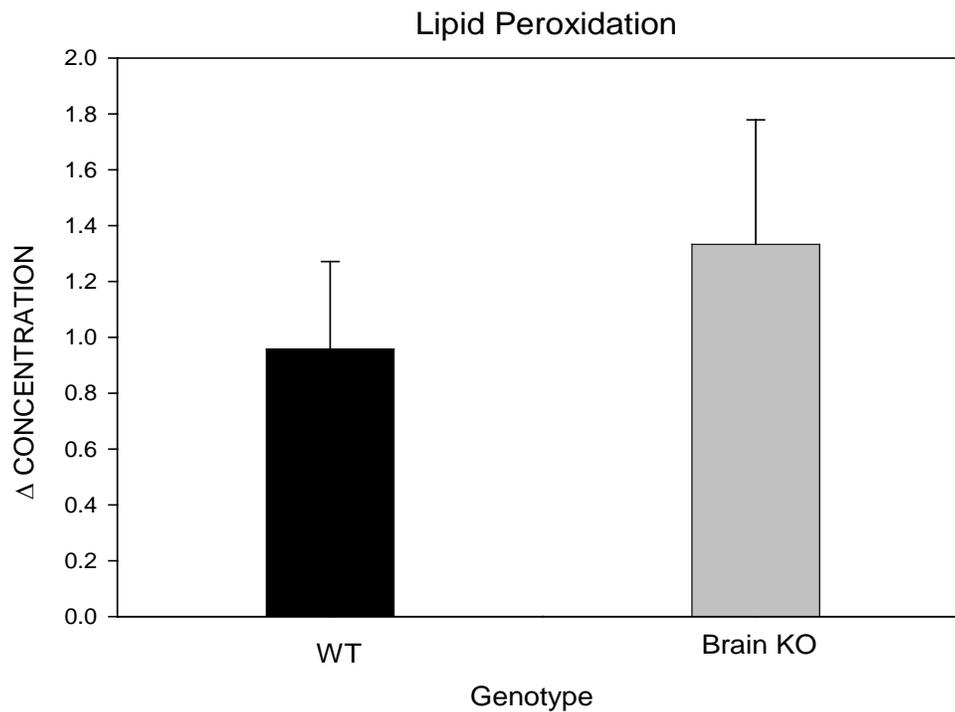
Figure 8. *Average levels of lipid peroxidation in microsome samples.* The levels of lipid peroxidation in the wild type and the brain knock-out samples were measured. The brain knock-out mice showed a slight elevation in the levels of lipid peroxidation, however the result was not statistically significant. Due to time constraints the levels of lipid peroxidation in the liver knock-outs was not measured.

DISCUSSION

The purpose of this study was to examine the levels and activity of CYP2E1 and CYP4A in mice that exhibited obesity in order to determine if these enzymes play a role in the development of NASH. With the use of BDNF knock-out mice, samples were obtained that exhibited age-related obesity and hyperinsulinemia, both of which are conditions that mimic the physiological states associated with steatohepatitis found in humans. Although the BDNF brain knock-outs exhibited steatotic livers, the pathology consistent with NASH was not established in these mice prior to testing.

Based on the metabolic conditions of the mouse BDNF brain knock-outs and the hypothesis that the increase of CYP2E1 levels may be a cause for the development of NASH, it was expected that the levels of this enzyme would be elevated in the obese mice in comparison with their non-obese counterparts. When the levels of this enzyme were measured it was seen that the levels of CYP2E1 were not significantly elevated.

In previous studies it had been established that CYP4A acts in an alternative biochemical pathway to produce NADPH-dependent lipid peroxidation activity in the absence of CYP2E1 [4]. Inspired by this finding, the levels of CYP4A were also measured in the obese mice and compared to their non-obese counterparts. The results from this measurement indicated that the levels of CYP4A in the obese mice were slightly elevated, but the samples exhibited a high degree of variability.

In order to confirm that the levels of enzymatic activity were proportional to the amount of enzyme present, enzymatic activity assays were performed. From these experiments mixed results were obtained. The CZ assay gave results that were consistent with the western immunoblot data and showed that the enzyme activity levels were not elevated in the obese samples. The PNP assay, which many consider to be a better indicator of CYP2E1 activity, showed that the obese samples did have elevated enzymatic activity levels in comparison with the wild type counterparts. The levels of lipid peroxidation were only slightly elevated in the obese mice, which is consistent with the slight elevation of CYP's present in their systems.

The initial data from this study are inconclusive for establishing the connection between steatosis, CYP's and NASH, however there are many possibilities as to why these results were seen. One possibility is that the elevated levels of CYP's and lipid peroxidation seen in patients with NASH are a result and not a cause of the disease. As the pathology of NASH progresses it is possible that body responds to these symptoms with an increase in CYPs in an effort to reduce the amount of fatty acids in the liver and stop the disease from progressing, which would also result in an increase in lipid peroxidation. The literature however documents that high levels of oxidative stress are present prior to the development of NASH [10]. Based on these previous findings the oxidative stress produced by CYPs may potentially still be involved in the development of NASH and should not be discounted.

Another aspect to consider when looking at the results of this experiment is the number

of samples tested in each group. In both the wildtype and the brain knock-out group only

three liver samples were tested. Within these samples there was a high standard deviation

with some samples expressing high levels of both CYP2E1 and CYP4A and some

expressing very low levels of these enzymes. In order to gain a better sense of the true

levels of enzymes in these two groups it would be necessary to increase the sample size.

A larger sample size would decrease the effects of variation and give a more accurate

representation of average levels of these enzymes between the two groups. Fortunately

Dr. Maribel Rios sent additional samples of wild type, brain knock-out and liver knock-

out mice on which additional testing can be performed. In looking at the literature the

link between lipid peroxidation caused by CYP2E1 and CYP4A is strong and should not

be disregarded as a possible cause of the development of NASH. At this point further

experimentation is necessary to provide evidence of this link, but the resources for these

experiments are available and waiting to be explored.

Another curious result of the experiments performed in this study was the elevated levels

of CYP2E1 in the female liver knock-outs compared to the male liver knock-outs and the

wild type mice. It was found in previous studies that BDNF is involved in the function of

HSC's and the development of fibrosis and hepatocellular carcinoma, both of which are

states associated with NASH [13,14]. In the absence of BDNF it is possible that NASH

would be slower in developing and the normal lipid peroxidation functions of the liver

would continue uninhibited by deteriorating liver function. This is a possible explanation

for the high levels of CYP2E1 in the liver knock-outs, but the cause for the difference

between gender remains to be established. Again, only two samples from each gender were tested and it would be necessary to include more samples in order to establish a coherent pattern for this observation.

From this study it is observed that the levels of CYP2E1 and CYP4A were not significantly elevated in BDNF brain knock-out mice compared with wild type mice. From the comprehensive literature that exists with regard to NASH the true mechanism behind the pathology remains to be conclusively determined. Indeed, the clinical evidence supports the idea that a steatotic liver alone does not determine whether a person will develop NASH. Other factors such as rapid weight loss or type II diabetes, conditions that both increase CYP2E1 expression, have been implicated in its development. Although the BDNF brain deletions caused obesity and insulin resistance in the mice used in this study, they did not express elevated levels of CYP2E1 or CYP4A. In the Leclercq study, mice fed a methionine and choline deficient diet also expressed steatosis in the liver, however they actually exhibited weight loss in comparison with mice fed a control diet [4]. These mice exhibited highly elevated levels of CYP2E1 and CYP4A, which demonstrates the development of NASH is not solely determined by obesity, but is also affected by diet. It is possible that the method used to induce steatosis in the liver could be a determining factor in the development of NASH and it would be wise to do further studies possibly using ob/ob mice or fatty Zucker rats to observe the levels of CYP2E1 and CYP4A.

CONCLUSION

Although the mechanisms leading to NASH have yet to be determined, the body of information regarding this disease is growing. As the incidence of diseases related to lifestyle and obesity increase, it has become a race against time to elucidate their causes and attempt to halt the ever-growing number of cases. As this study indicates the causes of NASH are not singular, but rather multiple factors related to obesity and insulin resistance combine and result in the fibrosis, hepatocellular damage and inflammation that have become hallmarks of this disease. The lipid peroxidation produced by CYP2E1 and CYP4A may quite possibly play a role in the development of NASH, however further study into the levels and activity of these enzymes in various models is necessary to firmly establish this link. Further research into this area will prove invaluable to the future treatment and prevention of NASH throughout future generations.

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