UNDERSTANDING THE RELATIONSHIP BETWEEN SEX AND NON-ALCOHOLIC FATTY LIVER DISEASE

by

COLETTE N. MILLER

(Under the direction of CLIFTON A. BAILE & MARY ANN JOHNSON)

ABSTRACT

The female sex has an innate protection from many metabolic diseases including non-alcoholic fatty liver disease (NAFLD) most commonly attributed to 17β-estradiol (E2). The goal of this dissertation is to provide further evidence behind the relationship of sex and NAFLD risk, and to develop potential explanations for this protection. Childhood risk of NALFD can be influenced by maternal obesity and the pre-natal environment including hormones. To determine if early metabolic sex differences can be found, livers from post-natal day 21 mice from lean and obese dams were assayed. While both sexes responded similarly to many of the selected measurements, sex differences in genes related to insulin signaling (GLUT 4 and IRS1) and the endoplasmic reticulum stress response were detected. In an acute hepatic injury model, male and female rats were fed a high fat diet for 72 hours. In that time period a large sex difference emerged in expression of genes related to apoptosis, cell repair, and remodeling in the liver, such that females appeared better able to adapt to this high fat diet. This is consistent with other acute injury models that show that E2 induces a pro-inflammatory state that is necessary for survival during acute stress and injury. Lastly, to investigate the efficacy of phytoestrogens to prevent ovariectomy-induced NAFLD, a phytochemical blend of resveratrol, genistein, and quercetin
was fed to ovariectomized rats. Due to the lipolytic and anti-adipogenic properties of the selected compounds, an increase in lipid mobilization and hepatic fat accumulation was measured. Despite this, the phytochemical blend reduced apoptotic and fibrogenic gene expression. Furthermore, the high-dose blend reversed ovariectomy-induced increases in serum alanine aminotransferase. Lastly, taking into account the entirety of this work, we have developed three hypotheses by which the female sex may be protected from NAFLD: (1) an inherent difference in endoplasmic reticulum stress and insulin sensitivity at birth that is most likely epigenetically regulated, (2) a rapid and heightened response to acute hepatic injuries including short exposures to high fat diets, and (3) the anti-inflammatory and cytoprotective effects of estradiol and its mimetics.

INDEX WORDS: Estrogen, Non-alcoholic fatty liver disease, Sex differences, High-fat diet, Phytochemicals, Insulin sensitivity, Apoptosis
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DEDICATION

This dissertation is dedicated in loving memory of Dr. Clifton A. Baile who was a great mentor, father-figure, colleague and friend to us all. He will be sorely missed by all those who had the wonderful opportunity of knowing him.
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"The term estrogen derives from the Greek ‘oirsqor’ which indicates a dipterous horsefly; the same term was used by Homer in the Odyssey (XX, 300) with the sense of torment, sting, but also of inspiration."
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CHAPTER 1
INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common form of liver disease and incorporates a spectrum of conditions. The first condition is steatosis, the development of at least 10% lipid accumulation in the tissue. From there it can progress to non-alcoholic steatohepatitis (NASH) to eventual cirrhosis, with some cases (under 4%) progressing to cancer (1). While NAFLD is not one of the leading causes of hepatocarcinoma, it is becoming an increasing reason for liver transplants. Currently there are no FDA approved medical therapies beyond transplantations for patients with end stage liver diseases including NASH and cirrhosis. Furthermore, transplants are difficult to obtain. In 2010, 6,291 transplants were performed in the US out of the 17,000 patients on the wait list (2). With projections of liver disease set to increase over the next decade, it is imperative that the etiology of NAFLD is further researched to allow for the development of much needed pharmacological interventions.

Pathophysiology

The two-hit theory for NAFLD has been well described and thus is well accepted in the research (figure 1). The first-hit is the accumulation of disease-promoting lipid within the liver. The vast majority of this lipid is derived from mobilization of adipocyte stores; approximately 60% (3). Additionally, a major upregulation in hepatic de novo lipogenesis is also seen and suggested to contribute up to 26% of hepatic fat accumulation, which is compared to the 5% that is seen in healthy individuals.
The increase in lipid storage becomes toxic to the liver and can promote apoptosis of hepatocytes creating a toxic environment. This lipotoxicity (lipid-toxicity) promotes inflammatory signaling through a variety of mechanisms. Palmitate binds to toll like receptor 4 (TLR4), leading to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB). Additionally, increases in free fatty acids result in endoplasmic reticulum stress as they can disrupt endoplasmic reticulum fluidity and protein folding properties, as well as increase reactive oxygen species via overwhelming beta oxidation pathways in the mitochondria (4). Furthermore, kupffer cells (hepatic macrophages) are actively recruited to lipid-filled hepatocytes, further promoting inflammatory pathways in the tissue (5). Activation of these pathways also promotes apoptosis. Ultimately, these disturbances can lead to the recruitment of pro-fibrogenic mediators and can also cause tissue-specific insulin resistance.

![Figure 1.1 The 2 hit-theory of NAFLD.](image)

**Figure 1.1 The 2 hit-theory of NAFLD.** The progression of NAFLD is described as hepatic lipid accumulation (hit 1) and downstream inflammation which causes eventual fibrosis (hit 2).

Insulin resistance plays a major role in the development and/or maintenance of NAFLD. 70% of type 2 diabetics have fatty liver, thus supporting a major influence of insulin resistance.
of hepatic disease (6). Insulin resistance in the adipocyte results in the inability to suppress lipolysis and thus contributes to the efflux of fatty acids out of the fat stores and into the bloodstream, where it is deposited in hepatic tissue. Additionally, insulin resistance in the liver results in an upregulation of hepatic lipogenesis and most likely contributes to the 21% increases in lipogenesis seen in those with NAFLD (3). Free fatty acids, in particular palmitate, have downstream effects on NFkB and c-jun N-terminal kinase (JNK) which include serine phosphorylation of insulin receptor substrate-1 (IRS-1) and its subsequent inactivation and insulin resistance (figure 2). Furthermore, the upregulation of suppressor of cytokine signaling 3 (SOCS3), caused by NFkB activation, results in the reduction of IRS-1 concentrations by targeting IRS-1 for ubiquitin-mediated degradation (7). In a paradoxical manner, insulin resistance promotes lipotoxicity in which lipotoxicity promotes inflammation and insulin resistance, producing a viscous circle that maintains the hepatic diseased state.

Figure 1.2 Mechanisms that drive lipotoxicity. Free fatty acids (FFA) and tumor necrosis factor alpha (TNFα) are released in an endocrine-manner from adipocytes and in a paracrine-fashion from apoptotic hepatocytes. The activation of their accompanying receptors mediate inflammatory signaling that can induce insulin resistance, increased lipogenesis, and apoptosis.
Lastly, emerging research has identified that the in utero environment can impact the offspring’s chances of development of NAFLD during childhood. In human studies, infants born to obese mothers had a 68% increase in hepatic fat at birth (8). This increased hepatic fat accumulation that occurs in utero results in upregulated lipogenesis, oxidative stress, and inflammation within the first 15 weeks of life in the offspring (9). Similarly with the increases in maternal obesity, metabolic disease projections for children are also expected to rise. While little is known about the causative factors of the in utero environment on fetal steatosis, both immune dysfunction and epigenetics have been suggested to play a role (10, 11). It is important that research is conducted during this time point, as NAFLD during childhood may severely impact projected hepatic health through life.

**Obesity and NAFLD**

Similar to the US obesity rates, NAFLD is present within one third of the adult population (12). Within the morbidly obese, 90% of the population is estimated to have NAFLD. Thus, a strong relationship between total adiposity and hepatic adiposity exists. This increase in adipose tissue, specifically in the visceral depots, increases overall rates of lipid mobilization throughout the body (13). This causes increased hepatic lipid deposition at disease-causing rates. The pro-inflammatory properties of free fatty acids causes the lipotoxicity described previously. Adipokines also may play a significant role in both the development of hepatic insulin resistance and inflammation during obesity. Macrophage accumulation within adipose stores is a hallmark condition of obesity. These activated macrophages release pro-inflammatory cytokines including tumor necrosis factor alpha (TNFα) and leads to the low-grade systemic inflammation that is also
considered an obesity hallmark (14). Adipocyte-derived TNFα can activate hepatic inflammatory signaling cascades inducing de novo lipogenesis, inflammation, and apoptosis within the liver.

**Sex differences in liver disease**

Epidemiological data has demonstrated that males have a 2-3 times increased risk of development of many hepatic diseases compared to their female counterparts including NASH and hepatocarcinomas (15). Estrogen has been shown to be cellular protective in various models and diseases. In regards to the liver, estrogen deficiencies caused by ovariectomy or aromatase inhibition cause disease-promotion within the liver including enhanced lipid deposition and lipotoxicity (16). These increases can be overcome with estradiol replacement further supporting the protective effects of this hormone. Unfortunately the specific mechanisms behind the observed sex differences and the role of hormones have not been fully investigated. The literature review has been split into two sections to specifically target 2 components of NAFLD, lipogenesis and inflammation. The potential roles of estradiol in these disease-promoting pathways have been recently published and are included in this dissertation.

**An introduction to this dissertation**

While a link between hepatic health and sex has been identified both in animal models and humans, very little research exists on this topic and until 2013 no mechanistic studies have been performed. The purpose of this dissertation is to help fill the void in research in female animal and cell models. Understanding how estrogens can impact hepatic health allows us to identify pathways that can be targeted for future pharmacological studies. Furthermore, these data also provides a snapshot into what can be expected for women when they enter menopause,
and thus can investigate what can be done to protect the liver during this transition. The research presented in this dissertation follows females throughout their lifespan. Chapter 4 investigates early sex differences in offspring of obese mice, a short-term hepatic injury model is studied in adult male and female rats in Chapter 5, and lastly we investigate the ability of a phytoestrogen blend in the ovariectomy rodent model in Chapter 6. At the time of printing, we are currently investigating a potential mechanism by which estrogen may be protecting the liver in the human HepG2 cell model. Preliminary data are presented in Chapter 7.

References


4. Liu Q, Bengmark S, Qu S. The role of hepatic fat accumulation in pathogenesis of non-alcoholic fatty liver disease (NAFLD). Lipids in health and disease 2010;9: 42.


CHAPTER 2

REVIEW OF THE LITERATURE:

THE MEDIATION OF HEPATIC LIPOGENESIS THROUGH ESTROGENS

Abstract

Estrogens have been shown to protect against various diseases and disastrous metabolic consequences of poor diets. Although a large body of research demonstrates estrogen’s ability to control food intake, adipogenesis, and oxidative stress, research regarding the effects of estrogens on hepatic lipogenesis, steatosis, and non-alcoholic fatty liver disease is only now accumulating. Estrogen deficiency in both human and rodent models directly results in the upregulation of hepatic lipogenic signaling - in both serum and hepatic triglyceride content - which leads to the development of fatty liver. In all models, estrogen replacement completely reverses these outcomes. Similar to the endogenous estrogen hormone, plant-derived phytoestrogens also appear to have beneficial effects related to prevention of hepatic lipogenic signaling and steatosis in rodent models. Additionally, such compounds can completely overcome the hepatic consequences that result from estrogen deficiency. While published research strongly supports that estrogens, both endogenous and exogenous, can protect against hepatic lipogenic signaling that can contribute to the development of non-alcoholic fatty liver diseases and adverse weight gain, little research exists on elucidating the mechanism behind this protection. Various pathways have been suggested, including manipulation of both leptin and signal transducer and activator of transcription 3 (STAT3) signaling. However, the discovery of x-box protein 1 elicits the identification of another potential pathway through which estrogen may be working. While the supportive work is strong, further research is needed to determine the mechanism behind the protection by estrogens from hepatic lipogenesis and associated diseases.
Introduction

Loss of estrogen dramatically increases the risk for many diseases because of estrogen’s pervasiveness in a variety of tissues. Weight gain associated with menopause can result in a secondary response, increasing the risk of weight-related diseases like diabetes, cardiovascular disease, and non-alcoholic fatty liver disease (Carr 2003; Matthews et al 1989). While the environmental causes of weight gain can be managed through dietary modification and physical activity, there are few options for preventing age-related and hormonal changes in body composition. As more women enter menopause already overweight or obese, any further increases in weight that are difficult to control will only result in increased risk of disease and an increased demand on the health care system (Heymsfield et al 1994; Poehlman 2002). In aged populations, BMI corresponds greatly to increased health care costs. Obese adults aged 65-years and older have increased yearly Medicare expenditures compared to those who are overweight; approximately $571 more in those with class I obesity and $1,271 more in those with class II/III obesity (Onwudiwe et al 2011). With the baby boomer population entering retirement age, such numbers are expected to rise in the coming years.

Hepatic lipogenesis contributes significantly to whole body fat accumulation and metabolic diseases (Glimcher and Lee 2009). Previous research indicates that hepatic lipogenesis plays a part in development of obesity, and knockdown of these pathways can blunt weight gain (Birkenfeld et al 2011). Since today’s environment is one of caloric excess, in many individuals the conversion of carbohydrates to fat for storage is perpetually occurring. Because the liver is the first tissue in the body to come in contact with metabolites from much of what we eat, it is an important player in energy balance. While research investigating how estradiol impacts adiposity through controlling food intake and lipolysis is widespread, little research exists regarding its effects on
lipogenesis in the liver. Preliminary research demonstrates that estrogens downregulate hepatic lipogenesis; thus they are currently used in the pharmacologic treatment in males with aromatase deficiencies suffering from hypertriglyceridemia and hepatic steatosis (Maffei et al 2004). The following review will focus on the published literature demonstrating the ability of endogenous and phytochemical estrogens to prevent obesity through downregulation of hepatic lipogenesis. Additionally, potential mechanisms behind this regulation will be discussed.

**Sex differences in diet-induced obesity**

It has long been established that in animal models of diet-induced obesity, females display some protection from the mal-effects of high-fat diets, whereas males do not. The protection is robust and includes increased glucose and cholesterol control, and in some studies females report reduced serum triglycerides more than males (Hwang et al 2010). Ovariectomy (OVX) in animal models produces similar effects, inducing a rapid weight gain that can be attenuated and reversed through estrogen replacement (Asarian and Geary 2002). Estrogen is a potent anti-obesity agent in a variety of body systems, both central and systemic. Estrogen reduces food intake, increases spontaneous physical activity and has potent lipolytic effects on adipocytes (Asarian and Geary 2002; Shi and Clegg 2009). While the effects of estrogen in the hypothalamus and on the adipocyte are well established, limited research exists in other tissues such as the liver. A recent study shows that male rats given injections of 17β-estradiol (E2) had reduced levels of hepatic lipogenesis, suggesting that the anti-obesity mechanisms behind estrogen are farther reaching than previously demonstrated (Hewitt et al 2004).
**Estrogen regulation of lipogenesis**

Although the current knowledge base of estrogen regulation of hepatic lipogenesis is limited, some evidence suggests that the anti-obesity effects of estrogen include downregulation of hepatic lipogenesis. This has been demonstrated recently by Gao et al (2006) who found that 4 weeks of E2 supplementation in female ob/ob mice resulted in a significant downregulation of genes involved in hepatic lipogenesis. Microarray analysis indicated significant reductions in many genes, including fatty acid synthase (FAS), acetyl CoA carboxylase-1 (ACC1) and stearoyl CoA desaturase-1 (SCD1) (Gao et al 2006).

OVX in animal models removes the main site of production for the endogenous female sex hormones, allowing for a model of menopause but also for the investigation of the specific effects of both estrogen and progesterone in the female body. Various studies have demonstrated that ovariectomy increases the lipogenic capacity of the liver. Paquette et al in 2008 demonstrated that OVX in female rats resulted in increased gene expression of sterol regulatory binding protein-1c (SREBP-1c), SCD-1, and peroxisome proliferator-activated protein alpha (PPARα) compared to intact females. These increases were also accompanied by increased fat accumulation in the liver (Paquette et al 2008). In another study, pelleted E2 replacement (0.012 mg/d) in OVX rats provided a reduction in hepatic and adipose fat accumulation, SREBP1c, ACC1 and SCD1 gene expression, and reduction of proinflammatory markers, including interleukin-6 (IL6), inhibitor of nuclear factor kappa-B kinase unit beta (IKK beta) and nuclear concentrations of nuclear factor kappa-B (NFkB) (Pighon et al 2011). These results suggest a potential relationship between hepatic inflammation and lipogenesis in the E2-deficient female rat.
Estrogen sulfotransferase (EST), the enzyme responsible for estrogen inhibition, has been associated with hepatic lipogenesis. Tissue specific knockdown of the EST enzyme results in protection from lipogenic activity in the liver. This protection was due to maintenance of hepatic E2, as EST knockdown in male and OVX female rats did not result in reductions of hepatic lipogenic activity (Gao et al 2012).

Estrogen levels in males have also been shown to modulate hepatic lipogenesis. Deficiency in aromatase, an enzyme involved in the synthesis of E2 from androgens, causes increased hepatic lipogenesis and fat deposition. Two separate case studies in adult men have been published describing patients with a genetic mutation in the aromatase gene (Pura M 2003). Both patients reported metabolic syndrome accompanied by hepatic steatosis in addition to a variety of other complications. Treatment with testosterone in one patient resulted in furthering the severity of insulin resistance, whereas transdermal E2 treatment resulted in the reversal of both insulin resistance and the hepatic steatosis (Maffei et al 2004). Similar findings have been shown in animal models, attributing the loss of E2-mediated reductions in lipogenesis as a contributing factor to the complications of aromatase deficiency. The aromatase knockout (ArKO) mouse provides a good model of E2-deficiency in males which displays increased weight gain and changes in lipid metabolism, including increased post-prandial serum triglycerides and cholesterol, hepatic steatosis and insulin resistance (Takeda et al 2003). These results indicate an increased lipogenic capacity due to the loss of E2. This is in fact what is observed; ArKO mice display increased hepatic triglyceride concentrations and increased FAS expression. Additionally ACC gene expression may be elevated, however it did not reach significance in one study (p=0.096) (Hewitt et al 2004). Furthermore, treatment of ArKO mice
via an estrogen receptor agonist ameliorates the observed hepatic lipid accumulation and normalizes lipogenic gene expression to comparable levels to wild-type mice (Chow et al 2011).

**Previously proposed mechanisms**

Several mechanisms behind the observed suppression of hepatic lipogenesis by E2 have been proposed. Microarray studies suggest two potential sites of regulation, direct upregulation of the leptin receptor and signal transducer and activator of transcription-3 (STAT3) gene expression. Estrogen supplementation was associated with reduced lipogenic gene expression along with the leptin receptor and STAT3, both of which have been previously demonstrated to be genomically regulated by estrogen receptor alpha. Lastly, an additional protein (x-box binding protein or XBP1) has also been shown to have connections with both the estrogen receptor and lipogenesis. However, further research on the direct mechanism of estrogen-regulated reductions in hepatic lipogenesis has yet to be published.

**STAT3 directed mechanism**

STAT3 is a transcription factor closely associated with receptor kinases that mediate cellular signaling in response to ligand binding at the receptor. Upon ligand binding, a conformation change occurs on the receptor resulting in the activation of its associated protein kinase. The kinase will then phosphorylate janus kinase (JAK) which then associates with and phosphorylates STAT3. The activated STAT3 protein then undergoes dimerization and can then translocate to the nucleus to mediate gene expression. The JAK-STAT pathway is commonly associated with both cytokine and growth factor receptors. Because of the diversity of associated
receptors, STAT3 has the ability to modulate a vast number of genes involved with cell growth, survival, and death.

Liver-specific knockout of STAT3 in mice results in increased hepatic triglyceride content in addition to increased SREBP-1c gene expression, suggesting a STAT3-mediated mechanism in lipogenic regulation (Inoue et al 2004). Viral reintroduction of STAT3 was found to reverse these effects in mice. This purposed regulation of SREBP1c gene expression has later been attributed to a direct inhibitory role of STAT3 on the promoter of the SREBP1c gene (Ueki et al 2004). Lastly, estrogen receptor alpha has been shown to regulate STAT3 expression. The STAT3 promoter lacks an estrogen receptor response element; however estrogen receptor regulates gene expression through binding to regulatory STATs and the DNA bound activating protein-1 at their respective response elements (Gao et al 2006). By this roundabout mechanism, E2 is capable of inducing STAT3 gene expression, providing increased STAT3 levels that can bind to the SREBP1c promoter and prevent its transcription (Figure 1). This was recently demonstrated in pancreatic β-cells of mice with specific STAT3 deletion (Tiano and Mauvais-Jarvis 2012). In this study, the reduction of SREBP1c by estrogen receptor alpha was found to be dependent on STAT3; however authors also indicated the AMP-activated protein kinase pathway as another site of estrogen-mediated activity.

Leptin receptor directed mechanism

The leptin receptor is one of the JAK-STAT associated receptor kinases that were previously mentioned. Leptin receptor activation results in the phosphorylation of STAT3, which is then capable of inhibiting SREBP1c gene expression, thereby reducing the lipogenic capacity of the liver. Leptin resistance, either by intracellular signaling or by reductions in receptor expression,
thus has been associated with increased hepatic lipogenesis. Both the leptin receptor deficient mouse (db/db) and the leptin deficient mouse (ob/ob) present with increased hepatic lipogenesis, steatosis, and obesity.

Figure 2.1 Estrogen regulation of lipogenesis. Estrogens may reduce hepatic lipogenesis by regulating STAT3 activity, which inhibits SREBP1c transcription. Abbreviations: Acetyl CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), steryl CoA desaturase 1 (SCD-1), sterol regulatory binding protein-1c (SREBP-1c), signal transducer and activator of transcription-3 (STAT3), DNA bound activating protein-1 (DBAP1), estrogen receptor alpha (ERα), leptin receptor (Ob-Rb). The encircled plus refers to transcriptional upregulation caused by the estrogen receptor.

It has been established that the estrogen receptor modulates leptin gene expression in various tissues. Estrogen receptor alpha knockout mice display reduced hepatic leptin receptor expression, which also corresponds with increased hepatic lipogenic gene expression (Bryzgalova et al 2006). Estrogen treatment in the ob/ob mouse produces opposing effects. A reversal of the increased lipogenic gene expression and steatosis are observed (Gao et al 2006). However, authors of this study suggest that the E2-mediated reductions in hepatic lipogenesis is
most likely not due to impaired leptin signaling alone. In the ob/ob mouse, the leptin produced is a variant that cannot activate the receptor. While E2 does modulate leptin synthesis, the ob/ob mouse given E2 supplementation would still have impaired leptin signaling. Therefore, as authors suggested, a combination of both STAT3 and leptin modulation is most likely occurring (Figure 1).

**A novel XBP-mediated mechanism**

XBP1 is a novel protein that has recently been of interest in a variety of inflammatory-mediated diseases. In most tissues, XBP1 coordinates the unfolded protein response during endoplasmic reticulum stress and is necessary for cell survival. Deficiencies in XBP1 result in a variety of diseases including inflammatory bowel disease, Alzheimer’s and Parkinson’s Disease, and type 2 diabetes (Kaser et al 2008; Matus et al 2011; Ozcan et al 2004). However, prolonged activation of endoplasmic reticulum stress and XBP1 activity results in a shift to apoptosis of the cell.

**XBP1 activation**

Activation of XBP1 results from an unconventional splicing mechanism performed by inositol-requiring enzyme-1 (IRE-1). IRE-1 is an endoribonuclease that cleaves a 26 base pair fragment from the XBP1 mRNA between sites 531 to 556 (Yoshida et al 2001). The resultant mRNAs include the XBP1u (261 amino acids) and XBP1s (376 amino acids). XBP1 splicing results in a frameshift mutation that provides for a basic leucine zipper- DNA binding domain and a transactivation domain within the extended C-terminal region of the translated protein (Yoshida et al 2001). The splicing and subsequent mutation permits the transcriptional activity of the translated pXBP1s (p- delineates protein). The remaining pXBP1u translated protein is quickly
degraded; however recent work suggests that it may serve as a negative regulator of the unfolded protein response (UPR) by sequestering pXBP1s and targeting it for proteasomal degradation (Yoshida et al 2006; Yoshida et al 2009).

IRE-1 activation normally occurs due to an accumulation of unfolded and misfolded proteins within the endoplasmic reticulum lumen. An accumulation of these proteins often suggests that the cell is undergoing stress, including viral infection, nutrient deprivation and oxidative stress. The normal degradation mechanism within the endoplasmic reticulum becomes overwhelmed, resulting in the need for increased machinery to handle the unfolded proteins (Zheng et al 2010). Such proteins bind directly to IRE-1, resulting in its oligomerization and transphosphorylation of its kinase domain. This then results in the subsequent activation of the endoribonuclease on the cytosolic region of IRE-1 (Cox et al 1993; Gardner and Walter 2011). IRE-1 mediated XBP1 activation has been shown to be an important regulator of hepatic lipogenesis and potentially, adipogenesis (Glimcher and Lee 2009; Sha et al 2009). Some have suggested that saturated fat-induced activation of XBP1 is independent of endoplasmic reticulum stress (ER stress) and the UPR; however the consensus appears to be otherwise (Zheng et al 2010). Increased saturated fat consumption results in various changes to cell membranes, including that of the endoplasmic reticulum, affecting the overall composition and motility of the membranes. Saturated fatty acids are readily converted to cholesterols, which in large amounts can be incorporated within the endoplasmic reticulum membrane and lead to a depletion of ER-calcium stores (Feng et al 2003; Ron and Oyadomari 2004). As calcium is necessary for many cellular processes, including protein folding, reductions in calcium concentrations reduce the efficiency of protein folding within the endoplasmic reticulum lumen, thereby promoting ER stress (Di Jeso et al 2003; Hojmann Larsen et al 2001). Lastly, a recent study in macrophages
found that through toll-like receptor-4 (TLR-4) activation, both ER stress and XBP1 activation can be induced (Martinon et al 2010). As saturated fat has demonstrated the ability to bind to TLR-4, this provides another site for saturated fat-induced XBP1 activation (Milanski et al 2009).

**XBP1 and lipogenesis**

While XBP1 activation in many tissues is a necessary step for cell survival, in both adipose and hepatic tissue XBP1 has an alternative role in promoting lipogenesis (Glimcher and Lee 2009; Sha et al 2009). Pharmacologic activation of ER stress, and thus XBP1 activation, results in an upregulation of lipogenesis in hepatic cells, steatosis, and hepatic inflammatory signaling in mice (Lee et al 2012a; Lee et al 2012b). Mice with deficiencies in hepatic XBP1 had decreases in circulating triglycerides, free fatty acids, and cholesterol, in addition to reduced fat accumulation in the liver (Glimcher and Lee 2009). Additionally, pharmacologic inhibition of XBP1 activation in post-prandial environments resulted in a downregulation of lipogenic gene expression (Pfaffenbach et al 2010). Such positive metabolic outcomes in these mice are attributed to the transcriptional regulation of lipogenic genes by XBP1. XBP1 is a transcription factor for a variety of lipogenic genes including FAS, ACC, and SCD-1 (Ren et al 2012).

**Estrogen regulation of XBP1**

In 2004, an analysis of promoter regions for the estrogen receptors located a region on the XBP1 gene suggesting a potential relationship between E2 signaling and XBP1 expression (Wang et al 2004). Since then most studies have investigated the role of estrogen receptor alpha and XBP1 activity in E2-responsive breast cancers. However, two recent studies have shown positive
outcomes in hepatic tissue after E2 treatment that appears to be dependent on regulating XBP1 activity. E2 treatment prior to an induced hypotension model of trauma-hemorrhage in male rats resulted in increased survival and reduced ER stress compared to vehicle-treated controls (Kozlov et al 2010). In OVX mice fed a high saturated fat diet, increased activation of hepatic XBP1 was observed compared to sham, chow-fed controls (Fukui et al 2011). Again, as demonstrated previously, E2 treatment significantly reduced XBP1 activation and ER stress in this study.

Figure 2.2 A novel mechanism of estrogen regulation. Estrogens may be reducing hepatic lipogenesis by regulating the transcription of a potent transcription factor (XBP1) for various lipogenic genes. Abbreviations: Acetyl CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), steroyl CoA desaturase 1 (SCD-1), diglyceride acyltransferase (DGAT), diglyceride (DAG), triglyceride (TAG), endoplasmic reticulum (ER), inositol requiring enzyme 1 (IRE-1), x-box protein 1 (XBP1), x-box protein 1 spliced variant (XBP1s), estrogen (E2), sterol regulatory binding protein-1c (SREBP-1c). The encircled plus refers to transcriptional upregulation caused by the estrogen receptor.

Lastly, unpublished work from the University of North Carolina at Greensboro found a significant reduction in total XBP1 mRNA in female rats compared to males fed a high fat diet for 72 hours (Miller 2011). This finding was accompanied by no increase in fat deposition in the
female rats, whereas high fat diet resulted in increased fat gain in male rats. These findings will be further explored to determine if the female rats also had reduced hepatic lipogenic gene expression. Overall, early studies support a possible mechanism behind estrogen-induced reductions in lipogenesis that may be due in part to downregulation of XBP1 activity, which is outlined in figure 2.

Lastly, it is important to mention the potential of XBP1-based therapies in regulating hepatic lipogenic signaling. Silencing of XBP1 both in vivo and in vitro leads to reduced hepatic lipogenesis without any reports of toxicity (Ning et al 2011). However as XBP1 appears to be necessary for liver tumor cell survival, knockdown of this pathway may produce negative effects in regards to hepatocarcinoma treatment (Cusimano et al 2010). Therefore, the use of XBP1 drug therapy may be highly situational and would need to be addressed with caution.

Phytoestrogens and lipogenesis
Phytoestrogens are plant-derived compounds that have demonstrated estrogenic activities by binding directly to the estrogen receptors. Because of the potential risks of hormone replacement therapy for use during menopause, much interest has emerged in investigating phytoestrogenic-compounds to manage the increase in disease-risk caused by the loss of ovarian hormones (Rossouw et al 2002). Phytoestrogens bind to the estrogen receptors at a lower affinity than E2, and thus are suggested to be safer than traditional hormone therapy (Kuiper et al 1998).

Much like E2, phytoestrogens have also emerged as potential regulators of hepatic lipogenic signaling, although research is much more limited in this area. Dietary genistein, a phytoestrogen derived from soy, reduced hepatic steatosis in male mice fed a high fat diet for 12 weeks (Kim et al 2010). These findings are further supported through observed downregulation
of lipogenic genes in both human lung cancer cells and within the HepG2 cell line after treatment with genistein (Hess and Igal 2011; Shin et al 2007). An additional phytoestrogen, daidzein, has also been linked with reducing hepatic lipogenesis and steatosis in high fat diet-fed mice as observed by reduced lipogenic gene expression and lipid concentrations (Kim et al 2011).

Similar to the studies involving E2, the studies investigating phytoestrogens have yet to specifically target and study a mechanism behind their actions. Various pathways have been suggested, including inflammatory and insulin signaling, SREBP-1c processing, and regulation of the liver X receptor β (Kim et al 2010; Kim et al 2011; Shin et al 2007). Additionally, phytoestrogens, much like E2, have been demonstrated to affect XBP1 activity. In neuroblastoma cells, both genistein and diadzein reduced XBP1 expression and activity in an estrogen receptor-dependent fashion (Park et al 2009). This finding suggests that phytoestrogens might work in a similar fashion within the liver, but further research is necessary.

Conclusions

Women often gain weight during menopause because of the dramatic reduction of circulating E2, which may only exaggerate the rise in overweight and obesity particularly in older adults. Many of the anti-obesity effects of E2 have already been targeted for study, including its effects on appetite and on lipolysis in the adipocyte. However, the breadth of research on E2’s effects on hepatic lipogenesis pales in comparison. So far it has been suggested that E2 appears to downregulate hepatic lipogenesis in various animal models and in humans, while the mechanism has yet to be elucidated. Greater understanding of anti-obesity actions of E2 is needed. Further insight into the potential nutraceutical and pharmaceutical targets will not only help reduce the
prevalence of excessive weight and obesity in older women, but also improve associated health outcomes.

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References


CHAPTER 3

LITERATURE REVIEW:

ESTROGENS, INFLAMMATION, AND OBESITY: AN OVERVIEW

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Abstract
Emerging research has suggested that inflammatory stress may play a role in the development of obesity. Both the leptin and insulin receptor are sensitive to intracellular inflammatory signaling that can be stimulated through toll-like receptor 4 activation by saturated fat. Pharmacological intervention within this cascade often protects animals from becoming obese, thus highlighting inflammatory pathways as a possible site of study in the prevention of pathologic weight gain. It has been well established in animal models that females display a marked reduction in the susceptibility to weight gain on high-fat diets compared to males. In addition, it has been widely accepted that females are partially protected from inflammatory-related diseases. At the molecular level, this reduction in disease susceptibility has been suggested to be due to the anti-inflammatory properties of 17 β-estradiol. Through direct free radical scavenging, transcriptional regulation, and protein interactions, chronic exposure to estradiol can reduce systemic inflammatory stress. As the knowledge base continues to grow on the etiology of obesity, further research is needed on the precise molecular pathways that can be inhibited by estradiol. Understanding of such pathways may provide a basis for the future use of estrogen and its related compounds (daidzein, genistein, resveratrol) to prevent weight gain in peri- and post-menopausal females.

Introduction
Obesity is commonly recognized as a disorder characterized by a chronic inflammatory status (Kahn and Flier, 2000; Wellen and Hotamisligil, 2003; Wellen and Hotamisligil, 2005). This systemic inflammation renders those affected susceptible to developing co-morbidities such as the metabolic syndrome, cardiovascular disease, cancer, osteoporosis, and neurodegenerative diseases (Lehrke and Lazar, 2004; Hotamisligil, 2006). While the development of the related
diseases has been linked to obesity-initiated inflammation, emerging research is beginning to suggest that obesity in itself may also share its etiology in inflammatory stress. Several animal studies have shown that the supplementation of anti-inflammatories to a high-fat diet can effectively defend against excess weight gain (Jayaprakasam et al., 2006; Charradi, et al. 2011). However, because of the complexity of human obesity, how large of a role inflammation plays in the development of obesity is contested. Regardless of the conflict, the following review will summarize the inflammatory pathways that may lead to the development of obesity with specific interest in leptin and insulin resistance.

It is well accepted that females experience lower rates of inflammatory diseases and this significant difference in the inflammatory response of women compared to men has been suggested to be due to the anti-inflammatory properties of 17 β-estradiol. As research is ever increasing suggesting inflammatory signaling plays an important role in increasing the susceptibility to excess weight gain, the anti-inflammatory properties of estrogens emerge as a potential modulator of these cascades. In the following sections we will provide potential explanations behind the anti-obesity effects of estrogen in regards to inflammatory stress. Also, we will review phytoestrogens that have anti-obesity effects in part due to their anti-inflammatory properties.

**The role of leptin and insulin resistance in obesity**

Two hormones have been identified as potent regulators of appetite suppression, leptin and insulin. Both hormones are released in relation to adiposity providing the brain with the status of the body’s long-term energy storage (fat). Leptin is released directly from adipocytes in relation to the amount of subcutaneous adipose tissue (Clegg and Woods, 2004; Shi and Clegg, 2009).
While insulin has a primary role in managing blood glucose levels, it is also released in proportion to visceral adipose mass and can inform the brain of the body’s energy status over the long-term. If fat stores are high due to a positive energy balance, signaling will result to reduce food intake and increase activity, while the opposite occurs when fat stores are low. Another common characterization of obesity is the development of central leptin and insulin resistance (Berthoud and Morrison, 2008). Hyperleptinemia under obese conditions fails to suppress appetite or increase energy expenditure since the circulating leptin fails to reach the brain or leptin signaling is disrupted leading to a condition called leptin resistance (Enriori, Evans et al., 2006). Similarly, hyperinsulinemia, most commonly seen in obese patients, is manifested by decreased insulin-stimulated glucose transport resulting in part from impaired insulin signaling and from downregulation of glucose transporter, GLUT4 (Reaven, 1995). Development of central leptin and insulin resistance may only propagate the malfunctions in energy balance, leading to even more weight gain and further fat deposition.

The hypothalamus is the target destination for such hormones, and serves as the master satiety switch by decreasing or increasing energy consumption and output. Injury to this area, seen often from tumor growth or lesions, results in rapid weight gain caused by an insatiable appetite (Hetherington and Ranson, 1940; Anand and Brobeck, 1951; York and Hansen, 1998; Berthoud and Morrison, 2008; Vinchon et al., 2009). This thereby strongly supports the idea that interference with hormonal signaling in the hypothalamus can in itself cause obesity. Research has previously demonstrated that dietary saturated fat promotes central leptin and insulin resistance (Milanski et al., 2009). How and when this hormonal resistance is developed during the timeline of obesity development is still a major issue under investigation, however several studies have discovered that systemic leptin and insulin resistance can occur in as little as 72 h
after rodents are switched to a high-fat diet (Wang et al., 2001; Morgan et al., 2004; Boghossian et al., 2009; Clegg et al., 2011).

**Malfunctions in leptin signaling**

The leptin receptor is a transmembrane receptor that falls within the cytokine receptor family. Upon ligand binding, the receptor undergoes a conformational change resulting in its autophosphorylation by the protein janus kinase (JAK) and subsequent activation of signal transducers and activation of transcription protein (STAT) (Wauman and Tavernier 2011 paper for a more descriptive view of leptin receptor signaling). Activated STAT serves as a transcription factor and stimulates expression of proteins that result in the inhibition of appetite inducing neurons [neuropeptide Y (NPY) and agouti-related protein (AgRP)], enhancing activity of appetite suppressing neurons [proopiomelanocortin (POMC)], and increasing intracellular levels of suppressor of cytokine signaling 3 protein (SOCS3) (Gong et al., 2008).

Many cytokine receptors are thought to be auto-inhibitory due to their ability to increase SOCS3 protein levels (Fig. 3.1). SOCS3 binds directly to these receptors, including the leptin receptor, rendering JAK inactive and preventing phosphorylation of STAT (Fig. 3.2). Therefore increased cellular concentrations of the SOCS3 protein can lead to a reduction in leptin signaling. While SOCS3 becomes elevated through normal leptin receptor activation, as described above, levels of the protein are also increased during inflammatory stress, again through activation of cytokine receptors such as toll-like receptor 4 (TLR-4) (Fig. 3.3) (Zhang et al., 2008; Milanski et al., 2009). Through this pathway, it is thereby possible for inflammatory
signaling to inhibit the leptin receptor without the presence of obesity, hyperleptinemia, and over-activation of the leptin receptor (Yang and Hotamisligil, 2008).

Figure 3.1 Chronic inflammation disrupts hypothalamic leptin and insulin signaling. Leptin receptor results in SOCS3 gene expression (1), resulting in inhibition of JAK-STAT signaling (2). Leptin receptor is also inhibited through saturated fat-induced activation of NFkB, and downstream increases in SOCS3 (3). Insulin receptor is sensitive to SOCS3 (4), as well as JNK (5), both a consequence of saturated fat-induced NFkB activation.

Abbreviations: toll-like receptor (TLR), inhibitor of nuclear factor kappa subunit beta (IKKβ), nuclear factor kappa B (NFkB), c-jun N-terminal kinase (JNK), insulin receptor substrate 1 (IRS 1), insulin receptor (IR), suppressor of cytokine signaling 3 (SOCS3), leptin receptor (ObR), janus kinase (JAK), signal transducers and activators of transcription (STAT), tumor necrosis factor alpha (TNFα), interleukin 6 (IL6)

Malfunctions in insulin signaling

The insulin receptor is a transmembrane tyrosine kinase receptor that activates its intracellular substrate, insulin receptor substrate 1 (IRS-1), through phosphorylation on its tyrosine residues. Within the hypothalamus, IRS-1 triggers activation of mitogen-activated protein kinase (MAPK) signaling and transcription. Much like the leptin receptor, activation of the insulin receptor results in the inhibition of NPY/AgRP signaling, activation of POMC signaling, thereby resulting in a pro-anorexic state (Schwartz et al., 1992).
Another trait that the insulin receptor shares with the leptin receptor is its sensitivity to increased intracellular SOCS3 concentrations. SOCS3 binds directly to the IRS-1 marking it for ubiquitin-mediated degradation, reducing its active concentrations, and thereby blunting insulin-stimulated signaling and its downstream effects (Fig. 3.4) (Rui et al., 2002; Uekio et al., 2004). To be expected, cross-talk between these two receptors is mediated by SOCS3. Because intracellular SOCS3 is elevated through normal leptin signaling, the increase can result in IRS-1 degradation and reductions in insulin sensitivity. Because of the connection between these receptors, changes in leptin signaling will also impact the insulin receptor as well.

Lastly, saturated fat is able to induce the activation of nuclear factor kappa-B (NFκB) through direct stimulation of TLR-4 (Milanski et al., 2009). This also results in the activation of JNK, which phosphorylates IRS-1 on its inhibitory serine residues, preventing its activation when insulin receptor is stimulated (Fig. 3.5) (De Souza et al., 2005; Yang and Hotamisligil, 2008).

**Hypothalamic inflammatory signaling and obesity**

In a hallmark study by Zhang et al. 2009, it was determined that inflammatory signaling caused by saturated fat directly results in obesity in rats. Chronic inflammation activates NFκB signaling in the hypothalamus resulting in resistance to insulin and leptin. In contrast, suppression of inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) in the medial basal hypothalamus, or in hypothalamic AgRP neurons, reverses diet-induced obesity (Zhang et al., 2008; Kleinridders et al., 2009). The molecular mechanisms involved in these processes include SOCS3, suppression of NFκB, and inhibition of insulin and leptin signaling. Signaling by the IKKβ/NFκB pathway in the hypothalamus represents an important factor in obesity, and it has
been proposed that suppression of hypothalamic NFκB signaling may inhibit obesity and related
diseases (Zhang et al., 2008; Kleinridders et al., 2009). Additionally, obliteration of TLR-4, the
receptor target of saturated fat, protects rodents from diet-induced obesity (Tsukumo et al., 2007;
Davis et al., 2008). This is due to a direct reduction of activated IKKβ complex and maintenance
of central insulin sensitivity. Pharmacological inhibition of NFκB also produces the same results;
reduction of food intake due to restoration of insulin sensitivity previously blunted by high-fat
diet (Posey et al., 2009).

There are changes in energy balance that precede the onset of obesity when animals are
defed a HF diet. A possible causal factor is that inflammation leads to leptin and insulin resistance
(De Souza et al., 2005; Zhang et al., 2008). De Souza et al. found inhibiting inflammatory
signaling by blocking JNK activation reduces food intake and bodyweight in rats fed a high-fat
diet compared to controls. The authors suggested this is due to the restored insulin signaling that
occurs when inflammation is reduced (De Souza et al., 2005). Additionally, when inflammatory
cytokines are delivered into the hypothalamus during high-fat feeding, a robust excess amount of
weight is gained (Oh-I et al. 2010). This may be attributed to cytokine-induced activation of
NFκB because the use of IKKβ inhibitor PS1145 in Oh et al.’s study prevented additional weight
gain.

Lastly, it also important to briefly mention the role of immune cells in mediating the
inflammatory response and obesity. Saturated fat has shown to stimulate immune cell infiltration
in a variety of tissue types including hepatocytes and adipocytes (Kennedy et al., 2008; Csak et
al., 2011). By reducing the functioning of infiltrating immune cells, this provides an additional
site of potential interventions. Studies that have blunted such functioning have found a protection
from excessive weight gain in animals fed a high-fat diet (Weisberg et al., 2006; Poggi et al.,
2011). Most recently, transgenic mice deficient in CD40L were protected from diet-induced obesity in addition to other metabolic consequences (Poggi et al., 2011). While research is quite limited in this area, the emerging literature is building a strong case that inflammation indeed plays a significant role in the development of obesity.

**Estrogens as anti-obesity agents**

It has been widely documented that in diet-induced obesity models, females gain weight at a slower pace than aged-matched males (Hong et al., 2009). Hong and colleagues reported that female mice gain less after 20 weeks on a high-fat diet than males. Additionally, the body weight change in HF diet-fed females mimicked that of chow-fed males. Research has shown that this is due to overall reduced energy consumption and increased levels of spontaneous physical activity (Basterfield et al., 2009; Priego, et al. 2009; Shi and Clegg, 2009). In addition, females tend to store fat in the less stressing subcutaneous depot compared to the visceral depot that has been associated with increased risk of disease (Clegg et al., 2006; Shi and Clegg, 2009).

The estrogen receptors (ER) are expressed in nearly every tissue in the body, with ERα being the most widely expressed and ERβ much more sporadic and only found in the hypothalamus, lungs, and female reproductive tract (Couse et al., 1997). Their anti-obesity effects can be attributed to its effects both systemically and centrally. 17β-estradiol (E2), the primary circulating female sex hormone, has shown to increase lipolytic activity of adipose, reduce levels of de novo lipogenesis, and regulates central leptin sensitivity by increasing leptin receptor expression, to name a few (Hewitt et al., 2003; Rocha et al., 2004; Bryzgalova et al., 2008; Shi and Clegg, 2009). For the purpose of this review however, more focus will be on estrogen’s roles within the hypothalamus regulating energy balance.
Anti-inflammatory activity of estrogens

E2 is involved in both the acute and chronic phase responses in regards to inflammation (Straub, 2007). Within the acute-phase response, E2 rapidly increases inflammatory signaling to help defend against bacterial or viral infection. Naturally, this would be a protective response and would increase the likelihood of survival during infection. However, chronic activation of pro-inflammatory environments results in an over-stressed system and deterioration of normal tissue responses. A chronic exposure to estrogen results in the blunting of inflammatory pathways, reducing the amount of damaged caused by long-term exposures to stressors such as dietary saturated fat or high glucose levels. It is by this chronic response that the female sex is protected from the development of diseases that share their etiology in inflammatory stress.

Estrogens, both biologic and environmental, bind to the estrogen receptors. (Vegeto et al., 2001; Vegeto et al., 2003; Vegeto et al., 2006). Estrogen receptor α (and in some cases ERβ) is expressed in immune and cytokine-producing cells including macrophages and microglia, and in vitro studies have shown E2-activated estrogen receptor α decreases pro-inflammatory cytokines (Vegeto et al., 2001; Vegeto et al., 2003). It should be noted that immune cells like macrophages that infiltrate adipose tissue are responsible for apparent increase in the production of proinflammatory mediators (Wellen and Hotamisligil, 2003). Tumor necrosis factor alpha (TNFα) is the predominant factor that mediates the crosstalk between macrophages and adipocytes and elevated systemic TNFα levels are found in obese individuals (Suganami et al., 2005). On the other hand, leptin induces the release of TNFα from mononuclear cells in circulation and estrogens inhibit leptin induced TNFα production (Fazeli et al., 2004). The anti-inflammatory properties of E2 can also be partially explained by the ability of estrogen receptors to act as
transcriptional repressors by inhibiting the activity of NFκB through protein–protein interactions between agonist-bound estrogen receptor and a subunit of activated NFκB (Stein and Yang, 1995; Ghisletti et al., 2005; Kalaitzidis and Gilmore, 2005). E2’s inhibitory action on NFκB function is still not clearly understood and may be target and gene selective (Harris et al., 2003; Chadwick et al., 2005; Kalaitzidis and Gilmore, 2005).

It is possible that E2 may be protecting normal hypothalamic signaling and energy balance by acting as an anti-inflammatory agent in the cell. Proestrus E2 levels are associated with reduced levels of inflammatory cytokines including TNFα, interleukin 6 (IL-6), and interleukin 8 (IL-8) (Straub, 2007; Hamilton et al., 2007). In addition to changes in serum cytokines during the estrous cycle, ovariectomy is associated with increased cytokine expression that is reversible upon E2 treatment (Evans et al., 2001; Hamilton et al., 2007). E2 has also been suggested to have antioxidant capacities by regulating gene expression of γ-glutamylcysteine synthetase, the rate limiting enzyme of glutathione synthesis, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase thereby increasing the cellular capacity of free-radical scavenging and reducing formation of reactive oxygen species (ROS), respectively (Fig. 2.1) (Straub, 2007).

Additionally, there is much research on the ability of E2 and estrogen receptor α to regulate NFκB activity (Stice and Knowlton, 2008). Genomically, E2 is able to increase the expression of nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (IkBα), the inhibitory subunit of NFκB (Fig. 2.2). E2 can also reduce IkBα phosphorylation, keeping it bound to NFκB and preventing the activation of NFκB (Fig. 3.3). Estrogen receptor α has the capacity to colocalize with the p65 subunit of active NFκB, preventing its transcriptional activities, which has been observed in rodents.
Figure 3.2 Anti-inflammatory effects of estrogens and phytoestrogens. Estrogens are capable of reducing ROS concentrations through direct free radical scavenging and ERα-mediated increases in other scavenger proteins (1). Intracellular reductions of ROS would reduce cellular stress and subsequent inflammatory signaling. ERα also mediates NFκB activity through increasing the inhibitory subunit IκBα (2) and direct protein on protein interactions with the NFκB complex (3). It is also important to mention that the anti-obesity effects of estrogens are in part due to ERα-mediated suppression of adipogenic genes (4). Abbreviations: estrogen receptor alpha (ERα), reactive oxygen species (ROS), nuclear factor kappa B (NFκB), tumor necrosis factor alpha (TNFα), interleukin 6 (IL6), nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (IκBα), peroxisome proliferator-activated receptor gamma (PPARγ), ccaat-enhancer binding proteins (C/EBPs)

Anti-inflammatory and anti-obesity activity of phytoestrogens

Natural products with estrogenic activity have been used for some time now as potential anti-obesity agents, including genistein and resveratrol. Much like E2, phytoestrogens are phenolic compounds capable of direct free radical scavenging thereby reducing ROS production and downstream inflammatory signaling (Fig. 3). However, unlike E2, the phytoestrogens have generally weak affinity to estrogen receptors compared to E2, in particular ERα (Kuiper et al., 1998). As mentioned previously, because the α subtype is widely expressed, many of the anti-obesity effects of these compounds may be due to estrogen receptor-independent signaling.
Interestingly though, a recent study demonstrated an estrogen receptor-dependent effect of resveratrol to reduce hepatic IL-6 in a trauma-hemorrhage model (Yu et al., 2008). Additionally both genistein and daidzein have also been shown to prevent endoplasmic reticulum stress activation in neuroblastoma cells (Park et al., 2009). As endoplasmic reticulum stress is a known activator of both NFκB and JNK, this may indicate ability for the phytoestrogens to reduce inflammatory signaling within the brain in an estrogen receptor-dependent fashion (Kaneko et al., 2003). Furthermore, in human adipocytes, anti-adipogenic effect of genistein was associated with decreased ERβ, which has an adipogenic role in adipose tissue (Park et al., 2009).

Figure 3.3 Molecular structure of estrogen and common phytoestrogens. Anti-inflammatory properties of the estrogens are in part due to the free-radical scavenging capabilities of the hydroxyl (OH) groups in their structures. Reduction in free radicals and reactive oxygen species result in reduced cellular stress and inflammatory signaling.

The key mediators of the inflammatory response, TNFα, IL-6, and cyclooxygenase (COX-2) are reduced by resveratrol in adipocytes (Gonzales and Orlando, 2008). It is well known that TNFα mediates its effects on adipocytes by activating the NFκB signaling and NFκB activity is elevated during adipocyte differentiation. Resveratrol is a potent inhibitor of NFκB activation indirectly influencing the adipocyte differentiation (Gonzales and Orlando, 2008). Further, in a recent in vivo study by Kim el al, rats fed with high-fat diet supplemented with resveratrol had lower body-weights and smaller adipose tissue depots accompanied with reduced expression of the pro-inflammatory cytokines, TNFα, interferon alpha (IFNα), interferon (IFNβ), and IL-6 in adipose tissue (Kim et al., 2011) suggesting that resveratrol, like E2, protected rats
from high-fat diet-induced inflammation and obesity.

**Conclusions**

As rates of overweight and obesity continue to rise, with some estimates suggesting 86% of the US population by 2030, a strong understanding behind the pathways that are affected during the development of obesity is needed (Wang et al., 2008). The increase of obesity and the diseases that make up the metabolic syndrome has resulted in a significant burden that is being placed on the health care system that will only continue to become more taxing in the coming years (Clegg and Woods, 2004; Haslam and James, 2005). As inflammation emerges to be a contributing factor to the etiology of obesity, such pathways provide a potential target for pharmacological intervention.

Many observational studies support that once someone becomes obese, their likelihood of sustaining a weight-loss over time is very low. This would suggest that the optimal way of reducing obesity and its comorbidities is to prevent it instead of treating it. The anti-inflammatory properties of estrogens, both biologic and environmental, provide insight on potential sites of intervention in inflammatory pathways that may reduce the burden on the body caused by westernized diets. Further research is needed in this area.

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References


receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology, 138(11): 4613–4621


CHAPTER 4

THE EFFECT OF AN IMMUNOMODULAR GLYCAN ON EARLY HEPATIC METABOLIC FUNCTION IN MALE AND FEMALE OFFSPRING OF OBESE DAMS\(^3\)

Abstract
Maternal obesity and overnutrition can imprint an increased risk of metabolic diseases, including non-alcoholic fatty liver disease, in the offspring. Emerging data has indicated that metabolic differences may also be affected by sex and placental transfer of inflammatory cytokines. Thus, the aims of this study were to investigate the effects of an immunomodulatory glycan (LNFPIII) on programmable hepatic metabolic pathways in both sexes of offspring from obese, high fat-fed dams. Livers were taken from post-natal day 21 C57BL/6 mice from dams fed either a low fat (4.3%) or a high fat diet (HFD) (35%). A subset of HFD dams were given a biweekly injection of the glycan (n=22). Histological analysis and hepatic triglyceride assays confirmed increased hepatic lipid deposition in offspring from HFD-fed dams (p<0.02). HFD caused increased SREBF1 and PPARγ expression in both sexes of offspring (p<0.02). Females from LFD-fed dams had a higher expression of SLC24A compared to all groups (p<0.03). Additionally in females HFD resulted in an increase in IRS1 expression (p<0.02), which was not observed in males. While HFD failed to increase hepatic GPT2 and spliced XBP1 mRNA, glycan treatment caused reductions in both genes in females only (p<0.05). Data from this study presents evidence that glycan LNFPIII protects female offspring from obese dams from metabolic injury due to early sex differences in insulin signaling and hepatic stress.

Introduction
Maternal high fat diets have been shown to influence the risk of obesity and metabolic diseases, specifically non-alcoholic fatty liver disease (NALFD), in the offspring of various species (1, 2, 3). NAFLD is the third most prevalent obesity co-morbidity affecting 20-30% of adults in Westernized societies (4). What is more concerning is the increasing trend of NAFLD in
children. Autopsy reports suggest that up to 9% of US children may have NAFLD, with 27% of these children experiencing non-alcoholic steatohepatitis and 9% experiencing fibrosis (5). Obese children are more likely to become obese adults; therefore children diagnosed with NAFLD will most likely have worsening liver conditions throughout their lifespan. Research has indicated that maternal programming of the offspring may influence the risk of early metabolic diseases, however little information is known on the mechanisms behind this programming. Thus furthering research in how the pre-natal and early infancy environment shapes the childhood-risk of disease is needed, particularly in the area of fatty liver.

The pathophysiology of NAFLD has been described as a 2 hit response that starts with increased hepatic lipid deposition (hit 1) and subsequent inflammation (hit 2), which causes downstream hepatic damage, fibrosis, and death. While it may be difficult to regulate the first hit, particularly in the offspring of obese mothers, it may be possible to defend against the pro-inflammatory hit 2 that can cause damage. The immunomodulatory glycan (LNFPIII) has already demonstrated the ability to reduce hepatic steatosis in diet-induced obese mice through modulating both hepatic lipogenesis and inflammation (6). LNFPIII increases the anti-inflammatory cytokine IL-10 capable of blocking nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and can inhibit the pro-inflammatory response to bacterial LPS. A relationship between IL-10 and hepatic steatosis has been previously established in various rodent models and within certain human populations (7, 8).

Breastfed infants have lower rates of NAFLD compared to their formula-fed counterparts (9). LNFPIII is present in human milk and considering its anti-inflammatory properties may serve as an extremely important modulator of later life metabolic outcomes in children (10).
Delivery of this compound to obese mothers may not only reduce their pro-inflammatory state, but may also prevent hepatic dysfunction by acting specifically in the offspring.

Lastly, a sex difference in childhood NAFLD occurrence has been observed, with boys showing higher incidences than in girls (11). It is possible that females are less sensitive to lipotoxicity and inflammatory stress than males, leading to a reduced risk of NAFLD in female offspring of dams fed a high fat diet. The proposed study will assess if sex differences exist in the efficacy of the aforementioned glycan in the risk of NAFLD development in offspring of obese dams.

**Methods**

*Animals and Housing*

Female C57BL/6 mice (6-7 weeks old) were purchased from Harlan Labs (Indianapolis, IN). Mice were housed 4-5 per cage and were given a 1 week acclimation period before entering into the study. After 1 week, mice were randomly assigned to either a low-fat diet (LFD; 4.3% fat, D12405J, Research Diets, Inc., New Brunswick, NJ; n=22) or a high-fat diet (HFD; 35% fat, D12492 Research Diets, Inc.; n=42) (Table 4.1). Mice were maintained on their respective diets and provided *ad libitum* access to food and water. The University of Georgia Institutional Animal Care and Use Committee approved all protocols prior to the start of this experiment.

**Table 4.1 Diet Composition.** Adult female mice were randomly assigned to either a LFD (D12450J) or a HFD (D12492) (Research Diets, Inc.) throughout the duration of the study. Post-natal day 21 offspring of obese and non-obese dams were used in this study. Abbreviations: low-fat diet (LFD) and high-fat diet (HFD).
Breeding and Glycan Treatment

After 6 weeks of feeding, mice were mated with control C57BL/6 males maintained on standard laboratory chow. Plug-positive females were individually housed during the duration of pregnancy and lactation, continuing to be fed their assigned LFD or HFD. Furthermore, at 6 weeks, female mice in the HFD group were randomly assigned to receive treatment of the immunomodulatory glycan (LNFP III- 25 µg) or its dextran vehicle (12). Glycan and vehicle treatments, given to both the control HFD and LFD mice, were delivered subcutaneously twice per week, beginning the day before initial mating and continuing through weaning. One male and one female mouse was randomly selected from each liter to obtain experimental groups (n=7-12 per group).

Hepatic Gene Expression

At post-natal day 21, the offspring of the dams were euthanized and tissue samples were weighed and snap frozen in liquid nitrogen following dissection. Isolation of mRNA was performed using the Qiagen MiniKit (Valencia, CA) following manufacturer’s instructions. The concentration of

<table>
<thead>
<tr>
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<tr>
<td><strong>Energy (%)</strong></td>
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<tr>
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<td>20</td>
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<tr>
<td><strong>Kcal/g</strong></td>
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<td>5.24</td>
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</table>
the isolate was determined using the NanoDrop 8000 spectrophotometer (Wilmington, DE).
cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit by
Applied Biosystems (Grand Island, NY) using 2 micrograms of the isolated RNA. Quantitative
PCR was run to determine differences in selected genes using the Applied Biosystems Sybr
Green master mix on the 7500 Prism system (Applied Biosystems, Grand Island, NY). Primers
were designed using PubMed databases and purchased from Integrated DNA Technologies (San
Diego, CA) and are listed in Table 2.

<table>
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<th>Anti-Sense</th>
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<td></td>
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<td></td>
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<td>SREBF1</td>
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**Hepatic Protein and Alanine Aminotransferase (ALT) Assay**

Frozen hepatic tissue was homogenized in modified RIPA Buffer containing 10% protease
inhibitor (Sigma-Aldrich, St. Louis, MO). Briefly, the homogenate was twice spun at 1,200 rpm
for 10 minutes and the supernatant was collected and stored at -20°C. The BCA Protein Assay
Kit (Thermo Scientific, Rockford, IL) was used to quantify total protein using manufacturer’s instructions. Hepatic ALT concentrations from the protein isolate were quantified using Point Scientific ALT Reagent (Canton, MI). Absorbance was read on 96-well plates using the FlexStation 3 (Molecular Devices, Sunnyvale, CA). Final ALT concentration was adjusted to the total protein content of the sample.

**Hepatic Triglyceride Accumulation**

The total lipid fraction from whole tissue was isolated using the Folch Method as described previously (13). Briefly, a 15-20 mg liver sample was homogenized in a 2:1 chloroform and methanol solution. Samples were incubated overnight at 4°C. The following day, samples were centrifuged at 2,000 rpm for 15 minutes. The supernatant was pipetted into a fresh microcentrifuge tube and washed with 1% NaCl. The mixture was vortexed and centrifuged at 2,000 rpm for 10 minutes. The subsequent top layer was discarded and the bottom layer was dehydrated and resuspended in 1% Triton X-100. Isolated triglycerides were quantified using the colorimetric Triglyceride (TAG) Reagent (Pointe Scientific, Canton, MI). Absorbance was read using the FlexStation 3 system (Molecular Devices, Sunnyvale, CA) and final TAG concentrations were determined using manufacturer’s instructions.

**Histology**

Hepatic samples were frozen on dry ice at the time of sacrifice and stored at -80°C until use. Hepatic sectioning was performed using the Leica 3050M cryostat-microtome at 6 µm. The liver samples were stained using Oil Red O with hematoxylin as a counter nuclear stain. Hematoxylin
and eosin (H&E) staining was performed at the UGA College of Veterinary Medicine Pathology Laboratory. The representative images in this manuscript were imaged at 100x magnification.

**Statistics**

Statistics were performed using STATISTICA software (version 7.0; Tulsa, OK). Two 2-way analysis of variance tests were used to determine significance of differences between main effects of diet and sex, and between sex and glycan treatments in the HFD mice. Significance between individual treatments was determined using Fisher’s Least Significant Difference test.

**Results**

*Offspring Characteristics*

Diet had a significant impact on body weight at post-natal day 21 (p<0.02; F(7.40, 1)). Male offspring of HFD-fed dams had a higher body weight than their LFD controls (p<0.02) (Table 4.3). Interestingly this increase was not observed in the HFD female offspring, as LFD and HFD female offspring were not significantly different. There was no significant effect of glycan treatment in the offspring, thus, body weights were similar to those of the offspring from HFD-fed control dams.

A significant sex effect was found in hepatic tissue weight between offspring from LFD and HFD-fed dams (p<0.03; F(5.20, 1)). Females had lower hepatic tissue weight compared to males. A significant glycan effect was found between HFD offspring (p<0.03;F(5.23,1)). Also in males from dams fed HFD, glycan treatment of the dams led to a reduced hepatic weight.
compared to male offspring from HFD-fed control dams (p<0.03) (Table 3).

**Table 4.3 Offspring characteristics.** Female C57BL/6 mice were fed either a LFD or a HFD. After 6 weeks of feeding a subset of the HFD mice were given an immunomodulatory glycan (LNFPIII). Male and female offspring of all treatment groups were euthanized at post-natal day 21 and included in the analysis of this manuscript. Data are presented as mean ± SEM values. Statistics were performed using ANOVA and Fisher LSD was used for post-hoc analysis. Means that do not share a common letter are statistically significant at p<0.05. Abbreviations: adjusted (Adj), low-fat diet (LFD), high-fat diet (HFD).

<table>
<thead>
<tr>
<th></th>
<th>LFD Males</th>
<th>HFD Males</th>
<th>LFD Females</th>
<th>HFD Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>8.50 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.66 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.37 ± 0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.32 ± 0.41&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>Liver weight (g)</td>
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<td>0.096 ± 0.00</td>
<td>0.093 ± 0.00</td>
<td>0.091 ± 0.00</td>
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<tr>
<td>Adj. liver weight</td>
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<td>0.0106 ± 0.00</td>
<td>0.01028 ± 0.00</td>
<td>0.01016 ± 0.00</td>
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<th></th>
<th>HFD Males</th>
<th>Glycan Males</th>
<th>HFD Females</th>
<th>Glycan Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
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<td>9.05 ± 0.28</td>
<td>9.32 ± 0.41</td>
<td>9.25 ± 0.22</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>0.096 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.089 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.091 ± 0.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.090 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adj. liver weight</td>
<td>0.0106 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0099 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01016 ± 0.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.0099 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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**Hepatic Steatosis**

Oil Red O staining of representative hepatic samples showed a large increase in lipid accumulation caused by a HFD (Figure 1A). The treatment of glycan in the HFD-fed dams, was unable to prevent this deposition in the offspring. Furthermore, lipid droplets can be identified within the H&E stains of the HFD and glycan mice.
To confirm histological analysis, hepatic TAG were isolated and quantified. A significant diet effect was measured (p<0.00; F(17.98, 1)). Both sexes of offspring from dams fed a HFD had increased hepatic TAG content compared to the LFD offspring (p<0.02) (Figure 1B). Similar to the histological assay, glycan failed to reverse the increase in TAG in either sex.

**Figure 4.1 Impact of glycan LNPFIII on hepatic steatosis.** Hepatic tissue of 21-day old offspring from dams fed either a LFD diet or HFD diet was isolated. A subset of dams from the HFD group were given a biweekly injection of the immunomodulatory glycan LPNFIII. (A) Pre-natal HFD exposure caused an increase in Oil Red O staining and lipid droplet formation observed in the H&E stain. (B) Isolated lipid from hepatic tissue was resuspended in Triton-X and assayed using a colorimetric assay for triglycerides. Statistics were performed using ANOVA and Fisher LSD. Means that do not share a common letter are statistically significant at p<0.05. Abbreviations: low-fat diet (LF) and high-fat diet (HF).

**Lipogenic Gene Expression**

Hepatic mRNA was isolated and gene expression assayed using qPCR. Changes in ACACA mRNA neared significance for diet effect at p=0.10, however a significant interaction was found for sex by glycan treatment (p<0.04;F(4.85,1)). There was a trend (p<0.06) for glycan treatment of dams decreased ACACA expression in female offspring but not male offspring (Figure 4.2A).
A significant sex by diet interaction was observed for FASN expression (p<0.02; F(6.92, 1)). HFD in dams increased FASN expression in female offspring from HFD compared to female offspring from LFD fed dams (p<0.05) (Figure 4.2B). A sex by glycan treatment interaction was also measured (p<0.05; F(4.22,1)); however, no individual group differences reached significance.

A significant effect of diet was measured for the upstream regulator of lipogenesis, PPARγ (p<0.04; F(5.04, 1), with PPARγ mRNA levels being higher in offspring from dams fed the HFD. No effect of the glycan treatment was observed. A HFD effect was additionally observed in SREBF1 expression (p<0.00; F(20.16,1)). Both male and female offspring from HFD-fed dams had an increase in SREBF1 mRNA compared to their representative LFD controls (p<0.03) (Figure 2D). Glycan treatment had no effect and thus is not presented.

Figure 4.2 Impact of glycan LNFPIII on lipogenic gene expression. Hepatic mRNA was isolated from post-natal day 21 offspring from dams fed either HFD or LFD. A subset of HFD-fed dams were given a biweekly injection of an immunomodulatory glycan (LNFPIII). Data were normalized to GAPDH. Statistics were performed using ANOVA and Fisher LSD. Means that do not share a common letter indicate statistical difference at p<0.05. Abbreviations: acetyl Co-A carboxylase alpha (ACACA), fatty acid synthase (FASN), peroxisome proliferator-activated receptor gamma (PPARγ), sterol regulator binding factor 1 (SREBF1), low-fat diet (LFD), high-fat diet (HFD).
**Insulin Signaling Gene Expression**

**Figure 4.3 Impact of glycan LNFPIII on insulin signaling.** Hepatic mRNA was isolated from post-natal day 21 offspring from dams fed either HFD or LFD. A subset of HFD-fed dams were given a biweekly injection of an immunomodulatory glycan (LNFPIII). Data were normalized to GAPDH. Statistics were performed using ANOVA and Fisher LSD. Means that do not share a common letter indicate statistical difference at p<0.05. Abbreviations: solute carrier family 2 (facilitated glucose transporter) member 4 (SLC2A4), insulin receptor substrate 1 (IRS1), insulin receptor (INSR), low-fat diet (LFD), high-fat diet (HFD).

For SLC2A4 (GLUT4) RNA expression, both sex and diet effects were observed, (p<0.04;F(5.08,1)) and (p<0.02;F(6.87,1)) respectively. Female offspring from LFD fed dams had a higher level of SLC2A4 mRNA compared to female offspring of HFD fed dams (p<0.04), while there was no difference between male offspring groups (Figure 3A). In addition, females had higher SLC2A4 mRNA levels compared to males, but it was mainly due to the large
difference between male and female offspring from LFD fed dams. Furthermore, an interaction for sex and glycan treatment was also seen (p<0.02; F(7.38,1)). However, individual group differences within sex didn’t reach significance.

A sex by diet interaction was found in IRS1 expression (p<0.02; F(6.19,1)). Interestingly, HFD in the dams led to an increase in IRS1 expression in females only (p<0.03); no other differences were observed (Figure 4.3B). A significant sex effect was observed in the glycan treatment analysis (p<0.05; F(4.23,1)); the only difference being between males from glycan treated dams and females from HFD vehicle-treated dams (p<0.05). Lastly, a significant sex by diet interaction was observed in INSR expression (p<0.02; F(6.06,1)). Males from HFD fed dams had lower INSR expression compared to males from LFD fed dams; there was no difference in females (Figure 4.3C). An effect of the glycan treatment was found (p<0.05; F(4.32,1)). In males from glycan treated dams, INSR mRNA was higher compared to males from HFD vehicle-treated dams (p<0.05).

**Endoplasmic Reticulum Stress Gene Expression**

There were no diet or sex effects on DDIT3 (CHOP) expression, however there was a significant glycan treatment effect (p<0.03; F(5.59,1)). Males from glycan-treated dams had a greater level of DDIT3 mRNA compared to the females from glycan-treated dams (p<0.02) (Figure 4.4A).

The spliced variant of XBP1 (XBP1s) was assayed to determine activation of the unfolded protein response arm of endoplasmic reticulum stress. A significant diet x sex effect was seen in XBP1s mRNA (p<0.04; F(5.03,1)). Females from LFD fed dams had lower levels of XBP1s mRNA than males from LFD fed dams. There was no difference between males and females from HFD fed dams. (Figure 4.4B). A significant sex by glycan treatment interaction was also
Female offspring from glycan-treated dams had reduced \( XBP1s \) compared to females from control dams (p<0.01), whereas glycan treatment of dams had no effect in males (Figure 4.4B).

*Expression of a regulator of DNA methylation*

Because of the widely hypothesized relationship between transfer of maternal stress onto the offspring epigenome, \( DNMT1 \) (DNA (cytosine-5-) -methyltransferases 1) was a chosen marker for alterations in epigenetic control. A significant ANOVA failed to be found in either the dietary analysis or glycan treatment analysis (Figure 4.5).
**Hepatic Function Assays**

ACTA2 RNA expression was measured due to its role in tissue fibrosis, however no significant effects of sex, diet, or glycan treatment were found (Figure 4.6A). A significant diet effect was observed in GPT2 expression (p<0.05; F(4.31, 1)). Male offspring from HFD-fed dams had reduced expression of GPT2 compared to offspring from LFD-fed dams (Figure 6B). A significant sex by glycan treatment was also measured in GPT2 expression (p<0.00; F(19.43, 1)). Glycan treatment of HFD fed dams increased GPT2 expression in male offspring (p<0.02), whereas, the opposite effect occurred in female offspring (p<0.02).

Lastly, hepatic ALT concentrations were determined from extracts of total hepatic protein. A significant effect of diet was observed (p<0.000, F(15.86, 1)). HFD in the dams resulted in an increase in hepatic ALT in offspring of both sexes, when compared to LFD-fed dams (p<0.05) (Figure 4.6C). In HFD fed dams glycan treatment had no effect in either male or female offspring.
Discussion

Maternal overnutrition causes increased hepatic lipid deposition in the offspring (14, 15, 16).

Consistent with previous studies, livers from HFD-offspring in our study had increased lipid accumulation and hepatic triglycerides. Similarly to other studies, the expression of major regulators of lipogenesis, including \textit{SREBF1}, were disturbed with maternal HFD exposure (16).

We did not investigate fatty acid oxidation in this study. However epigenetic reductions in PPAR
gamma coactivator 1 alpha (PGC1α) expression have been previously documented in a primate model of maternal overnutrition (15). Deficiencies in PGC1α impair mitochondrial biogenesis and fatty acid oxidation (17). Therefore it is possible that reductions in genes related to lipid utilization also occurred in our study. Furthermore, future work should also determine how sex can regulate these pathways, as recent work by Mischke et al have indicated this possibility (18).

In a 2007 study, although statistical analysis was not performed, investigators reported reductions in serum triglycerides and insulin in female offspring of HFD-fed dams compared to males (19). A sexual dimorphic insulin concentration was also seen by Sun et al (20); however, statistical analysis was not performed between sexes. Findings from these two studies suggest a sexual dimorphism in insulin sensitivity and signaling. In our study we found that females, particularly ones from LFD-fed dams, had a greater SLC2A4 expression compared to the males. Furthermore, IRS1 expression was increased in the female offspring from HFD-fed dams. In combination with potential differences seen in serum insulin in the aforementioned studies, it is possible that females may be protected from metabolic diseases including NAFLD through a beneficial insulin profile.

Sex specific differences in metabolic diseases have long been identified and attributed to the female hormone 17β-estradiol. Emerging research has demonstrated an inherent dimorphism in early metabolic diseases that is seen prior to puberty, thus removing a causative role of estrogens in this protection (18, 21, 22). It has been hypothesized that epigenetics may regulate both inherent sex differences in utero and early infancy, in addition to dimorphic responses to disease promoters. Interestingly, the placenta is highly adaptable to the maternal environment in a sexually dimorphic manner and may provide a mechanism behind early epigenetic modifications in the offspring (23). While we did not see any differences in DNMT1 expression
in our study, we do not negate the possibility of epigenetic regulation of the metabolic outcomes that were measured and the need for further research in this area.

An immunomodulating, anti-inflammatory glycan was used in our study to determine whether maternal HFD-induced inflammatory programming in the offspring could be prevented. A similar sexual dimorphic response was measured with maternal glycan treatment. The most significant contribution of the glycan was on the activation of endoplasmic reticulum stress. While in general females had less XBP1 splicing compared to males, females from glycan-treated dams had lower splicing females from vehicle treated dams (p<0.05). This is not surprising as endoplasmic reticulum stress is highly related to inflammation and lipotoxicity, and thus may indicate reduced hepatic stress in this group (24, 25). Furthermore, hepatic GPT2 expression was also downregulated in the female offspring of glycan-treated dams. Interestingly, the enzymatic ALT activity did not follow the same pattern as GPT2 expression. It is possible that the discrepancy between these two data points could be related to assay sensitivity. Enzymatic assays for ALT measures both isoforms of ALT, whereas our gene expression data reflects the isoform more associated with NAFLD (12). While a functional assay to determine hepatic ALT concentrations found no reduction with glycan treatment, the reduced GPT2 mRNA may be an indicator of reduced hepatic damage in this group.

Maternal obesity may impair the offspring’s ability to regulate energy in our current obesogenic environment, further exaggerating metabolic disease in adulthood (2, 27). Therefore it is necessary to determine mechanisms by which maternal transfer occurs and how it may be prevented. Our study provides evidence for a sex-specific response in an early programming model. From these data, we conclude that females may be at a reduced risk of childhood NAFLD due to enhanced insulin sensitivity and reduced hepatic stress compared to their male
counterparts. Furthermore, supplementation of an immunomodulatory glycan during gestation produced a sex-specific response in offspring suggesting a need for further research in this area.

References


CHAPTER 5

ACUTE EXPOSURE TO HIGH FAT DIETS INCREASES HEPATIC EXPRESSION OF GENES RELATED TO CELL REPAIR AND REMODELING IN FEMALE RATS

Abstract

High fat diets (HFD) promote the development of both obesity and fatty liver disease through the upregulation of hepatic lipogenesis. Insulin resistance, a hallmark of both conditions, causes dysfunctional fuel partitioning and increases in lipogenesis. Recent work has demonstrated that systemic insulin resistance occurs in as little as the first 72 hours of a HFD, suggesting the potential for hepatic disruption with HFD at this time point. The current study sought to determine differences in expression of lipogenic genes between sexes in 3 month old male and female Long-Evans rats after 72 hours of a 40% HFD or a 17% fat (chow) diet. Due the response of estrogen on hepatic signaling, we hypothesized that a sexual dimorphic response would occur in the expression of lipogenic enzymes, inflammatory cytokines, apoptotic, and cell repair and remodeling genes. Both sexes consumed more energy when fed a HFD compared to their low fat-fed controls. However, only the males fed the HFD had a significant increase in body fat. Regardless of sex, HFD caused downregulation of lipogenic and inflammatory genes. Interestingly, females fed a HFD had upregulated expression of apoptotic and cell repair-related genes compared to the males. This may suggest that females are more responsive to the acute hepatic injury effects caused by HFDs. In summary, neither male nor female rats displayed disrupted hepatic metabolic pathways after 72 hours of the HFD treatment. Additionally, female rats appear to have protection from increases in fat deposition, possible due to increased caloric expenditure; male rats fed a HFD were less active as demonstrated by distance traveled in their home cage.
Introduction

Hepatic lipogenesis contributes significantly to body fat accumulation and metabolic diseases [11]. Previous research has indicated that hepatic lipogenesis plays a part in the development of obesity, and knockdown of hepatic lipogenic pathways can blunt both steatosis and weight gain in non-human models [2]. With obesity rates expected to rise to 50% of US adults by 2030, contributing up to 8.5 million additional cases of diabetes, it is imperative that the metabolic disturbances that occur during the onset of obesity are delineated [34].

Recent studies have demonstrated that male rats fed obesogenic high fat diets (HFD) for 72 hours rapidly develop both insulin and leptin resistance prior to the development of obesity as defined previously [37]. Insulin resistance has been observed in the hypothalamus and the amygdala, two regions that regulate food intake behaviors [3; 7]. Insulin resistance has also been demonstrated in adipose and liver in as little as 72 hours; in correspondence with adiponectin resistance in muscle tissue [20; 22; 25]. Such animal models have temporary increases in whole body adiposity and hepatic triglyceride content, steatosis, and serum cytokine levels.

Hepatic insulin resistance results in alterations of normal fuel partitioning, including a marked inability to reduce gluconeogenesis while at the same time promoting lipogenesis [4]. HFDs promote the development of hepatic insulin resistance, observed to occur at 72 hours, which suggests the potential for increased lipogenesis at this time point. A recent study in male mice, however, indicated opposing results [25]. While the mice fed the HFD displayed both hepatic insulin resistance and steatosis, they did not show increases in lipogenesis. The potential explanation behind this observation was that hepatic lipogenic enzymes are still capable of responding to substrate-level inhibition due to the influx of dietary fat after 72 hours of a HFD.
Increases in hepatic fat content were attributed to increased fat deposition caused by the increased dietary fat.

Most studies investigating the metabolic changes that occur after 72 hours of a HFD have been in male models. Currently no research is available investigating potential sex differences. It has long been established that females display protection from the mal-effects of HFDs, primarily due to the widespread anti-obesogenic role of 17β-estradiol [29]. Preliminary research has demonstrated that estrogens down-regulate hepatic lipogenesis, and thus are currently used in the pharmacologic treatment in males with aromatase deficiencies suffering from hypertriglyceridemia and hepatic steatosis [10; 21; 24]. It is therefore possible that the observed protection from increased adiposity in females may be in part due to a sex difference in hepatic lipogenesis during the early exposure to a HFD. The current study sought to investigate potential sex differences in hepatic gene expression during this time period. Due to observed sex differences in non-alcoholic fatty liver disease, we hypothesized that a sexual dimorphic response would be measured in genes related to lipogenesis, inflammation, and cell injury in 72 hours of a HFD. To assess this, quantitative PCR was used to measure expression of genes in the above categories in adult male and female Long-Evans rats, a standard diet-induced obesity rodent model. Additionally, differences in body composition after 72 hours of a HFD were measured using dual X-ray absorptiometry.

**Methods and materials**

*Animals and housing*

Three month old male (n=15) and female (n=17) Long-Evans rats were purchased from Harlan Labs (Fredrick, MD). Upon arrival they were given 1 week to acclimate to the facility before
introduction to sex-specific colony rooms. Prior to the start of the experiment, rats were maintained on a standard laboratory chow (17% fat and 3.1 kcal/g, Harlan Teklad #7012; Indianapolis, IN) (Table 1). Rats had access to food and water *ad libitum* throughout the experiment. Rooms were temperature (22 ± 2 °C) and humidity controlled and kept on a 12:12 light/dark cycle. At the start of the experiment, within each sex rats were randomly assigned to HFD (40% fat and 4.54 kcal/g, Research Diets #D03082706; New Brunswick,NJ) or chow (low fat diet (LFD)) (Table 5.1). Food intake and body weights were measured at the start and end of the experiment. The University of North Carolina at Greensboro Institutional Animal Care and Use Committee approved all protocols for this experiment.

**Table 5.1 Diet composition**

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>LF diet</th>
<th>HF diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harlan Teklad #7012</td>
<td>Research Diets #D03082706</td>
<td></td>
</tr>
<tr>
<td>% kcal</td>
<td>% kcal</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>58</td>
<td>46</td>
</tr>
<tr>
<td>Fat</td>
<td>17</td>
<td>41</td>
</tr>
<tr>
<td><em>Saturated fat %</em></td>
<td>16</td>
<td>62.5</td>
</tr>
<tr>
<td><em>Monounsaturated fat %</em></td>
<td>26</td>
<td>30.6</td>
</tr>
<tr>
<td><em>Polyunsaturated fat %</em></td>
<td>58</td>
<td>6.9</td>
</tr>
<tr>
<td>Total isoflavones (mg/kg)*</td>
<td>300-600</td>
<td>0</td>
</tr>
<tr>
<td>Kcal/gm</td>
<td>3.41</td>
<td>4.54</td>
</tr>
<tr>
<td>Minerals</td>
<td>Ca (g/Kg)</td>
<td>10.0</td>
</tr>
</tbody>
</table>

1Three month old Male and female Long-Evans rats were fed diets either high in fat or low in fat for 72 hours.
2Harlan-Teklad 7012 is a natural ingredient diet. Major macronutrients come from corn, soybean, oat, wheat, and alfalfa ingredients.
3Protein source for the HF diet was casein and cysteine.
4Carbohydrate source for the HF diet was corn starch, maltodextrin 10, and sucrose.
5Fat source for the HF diet was butter with additional soy bean oil.
6Harlan-Teklad 7012 is an autoclavable diet, which result in some vitamin loss not reflected in this table.
Determination of estrous cycling

The estrous phase of each rat was determined daily by vaginal lavage as previously described by Becker et al [1]. Obtained vaginal cells were collected onto glass slides for examination of cell types under a light microscope. Samples were taken at the same time daily until the timing of 2 estrous cycles could be confirmed. When the timing of the estrous cycle was determined for each rat, the experiment was started so that they would be in proestrus on the day of sacrifice. Male rats were handled daily during this time period in order to prevent potential bias.
Spontaneous physical activity

Measurements of home cage behaviors were performed through real-time video surveillance through HomeCage Scan software (Clever Systems, Inc; Reston, VA). The room was set up with blue backgrounds and red lights for recording during the dark cycle. Animals were given 1 day acclimation to the behavioral room prior to the start of the study. Cages were changed daily in order to reduce the amount of potential interference around the rat. To provide an estimate of spontaneous physical activity, the distance traveled in each cage was obtained from the program.

Body composition

Because no non-invasive methods exist to quantify subcutaneous and visceral fat, post-mortem measurement of body composition was performed by dual X-ray absorptiometry. After sacrifice, the skin along with the attached subcutaneous fat (pelt) was dissected from the muscle wall and visceral fat (carcass) as previously described by Clegg et al [6]. Body composition was then performed using a GE Lunar Prodigy Advanced System (GE Healthcare; Milwaukee, WI) and the data were analyzed by Encore 2007 Small Animal software (version 11.20.068). Both the pelt and body were scanned in duplicate to determine body fat and lean body mass.

Hepatic gene expression

At sacrifice, a section of the liver was preserved in RNAlater and stored for 24 hours at 4 °C and then stored at -80 °C until processed. RNA was isolated using QIAGEN RNAeasy kits (Qiagen, Inc: Valencia, CA) according to the manufacturer instructions. RNA concentration and purity was assessed by Nanodrop spectrophotometer (Thermo Scientific, ND-1000; Wilmington, DE). 2μg of RNA for each sample was combined with RNase free H2O and master mix solution (Applied Biosystems; Foster City, CA) and run in a Thermocycler (Applied Biosystems; Foster
City, CA) for 2.5 hours to obtain cDNA. The collected cDNA was used to determine gene expression via qPCR for interleukin 6 (Il6), tumor necrosis factor alpha (Tnfa), suppressor of cytokine signaling 3 (Socs3), acetyl Co-A carboxylase (Acaca (Accl)), fatty acid synthase (Fas), sterol regulatory element-binding protein 1 (Srebf1 (Srebplc)), sterol Co-A desaturase (Scd1), diglyceride acyltransferase (Dgat1), peroxisome proliferator-activated receptor gamma (Pparγ), neuropeptide y (Npy), adipose triglyceride lipase (Pnpla2 (Atgl)), x-box binding protein (Xbp1), CAMP responsive element binding protein 1 (Crebl), janus kinase 2 (Jak2), mitogen-activated protein kinase (Mapk), signal transducer and activator of transcription 3 (Stat3), annexin 5 (Anxa5), Bcl-2 binding component 3 (Bbc3), caspase 2 (Casp2), mothers against decapentaplegic homolog 1 (Madhl (Smad1)), biglycan (Bgn), fibronectin (Fn1), GATA binding protein 3 (Gata3), matrix metallopeptidase 11 (Mmp11), serpin peptidase inhibitor clade H member 1 (Serpinh1 (Hsp47)), osteonectin (Sparc), and vascular endothelial growth factor (Vegf) using commercially available TaqMan Gene Expression Assays from Applied Biosystems. qPCR was performed using a 7900 HT system by Applied Biosystems (Table 5.2).

Table 5.2 Probes used in real time PCR

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Context sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous control</td>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Rn01775763_g1</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>18S</td>
<td>45S pre-ribosomal RNA</td>
<td>Rn03928990_g1</td>
</tr>
<tr>
<td></td>
<td>AcACA</td>
<td>Acetyl CoA carboxylase alpha (ACC1)</td>
<td>Rn00573474_m1</td>
</tr>
<tr>
<td></td>
<td>PNPLA2</td>
<td>Patatin-like phospholipase domain containing 2 (ATGL)</td>
<td>Rn01479969_m1</td>
</tr>
<tr>
<td></td>
<td>DGAT1</td>
<td>Diglyceride acyltransferase</td>
<td>Rn00584879_m1</td>
</tr>
<tr>
<td></td>
<td>FASN</td>
<td>Fatty acid synthase</td>
<td>Rn01463550_m1</td>
</tr>
<tr>
<td></td>
<td>Npy</td>
<td>Neuropeptide Y</td>
<td>Rn00561681_m1</td>
</tr>
<tr>
<td></td>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
<td>Rn00594894_g1</td>
</tr>
<tr>
<td></td>
<td>SCD1</td>
<td>Sterol CoA desaturase</td>
<td></td>
</tr>
</tbody>
</table>
**XBP1 splicing assay**

Quantification of spliced Xbp1 mRNA was performed using methods previously described by Hirota et al [14]. Briefly, cDNA was combined with PCR master mix and Xbp1 primer purchased from Applied Biosystems under manufacturer instructions. In order to create double stranded cDNA, this mix then underwent 2 PCR cycles in a thermocycler under the following conditions: 94°C 5 minutes, 95°C 30 seconds, 55°C 30 seconds, 72°C 30 seconds, 95°C 30 seconds, 55°C 30 seconds, 72°C 30 seconds. 0.75 U of PstI (Promega: Fitchburg, WI) was then added to each sample for 1 hour prior to quantitative PCR in a 7900 HT system.

**Statistical analyses**

mRNA samples from rats were selected for this study from a larger unpublished dataset that proved to not be outliers in their respective groups for food intake, body weight change, and body composition. If an animal was an outlier in any one of these measures, they were removed from the current study. Power analysis was performed using G*Power 3 statistical power
analysis software [9]. The final number of animals selected (n=32 from the original n= 48) for this study had appropriate power to maintain statistical significance in the above variables. Outlier tests were performed using the GraphPad QuickCalcs outlier calculator available online. Final statistical analysis was performed using Statistica (version 7.0) for Windows. Treatment effects and interactions were tested using two-way ANOVA, with the F-statistic and degrees of freedom provided. Individual group differences were tested using Tukey’s HSD. Data is presented as means ± SEM and significance was determined at p<0.05.

Results

72 hours of a HFD on food intake and body weight gain

HFD resulted in a significant sex by diet interaction in food intake measured by difference in food weight (p<0.00; F(17.83, 1)). There was no diet effect in males; however females fed a HFD ate more diet compared to the low fat-fed females (p<0.00) (Table 5.3). This hyperphagia in the females remained when food intake was normalized for body weight (p=0.01), however the male rats fed a HFD reduced their food intake compared to males fed the low fat diet (LFD) (p<0.00) (Table 5.3).

A significant sex by diet interaction was observed in caloric intake (p=0.02; F(6.43, 1)). Females on a LFD had reduced caloric intake compared to males fed LFD (p<0.00) (Table 5.3). However, this sex difference was attenuated on the HFD and both sexes significantly increased their caloric intake on the HFD compared to LFD controls (p<0.00). When caloric intake was normalized, a significant sex by diet interaction was seen (p<0.00; F(23.30, 1)). The increased caloric intake was lost in HFD-fed males, yet remained within the female diet groups (p<0.00) (Table 5.3).
The increase in caloric intake caused by a HFD resulted in increased body weight compared to LFD-fed controls (p<0.00; F( 24.93, 1)). However, only females on a HFD had a significant increase in body weight gain compared to controls (p<0.05) (Table 5.3). No difference in body weight was observed between male diet groups presumably due to inexplicable variances as demonstrated by unusually large standard errors in the male rats.

Table 5.3 Food intake and changes in body weight and body composition

<table>
<thead>
<tr>
<th></th>
<th>LF diet Males (n=6)</th>
<th>HF diet Males (n=9)</th>
<th>LF diet Females (n=8)</th>
<th>HF diet Females (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 h FI (g)</td>
<td>92.73 ± 2.98a</td>
<td>87.41 ± 2.43ac</td>
<td>61.40 ± 2.58b</td>
<td>78.18 ± 2.43c</td>
</tr>
<tr>
<td>72 h FI (g) / BW</td>
<td>0.26 ± 0.01a</td>
<td>0.21 ± 0.01b</td>
<td>0.21 ± 0.01b</td>
<td>0.26 ± 0.01a</td>
</tr>
<tr>
<td>72 h FI (kcal)</td>
<td>309.70 ± 14.44a</td>
<td>393.10 ± 12.21b</td>
<td>208.25 ± 12.21c</td>
<td>354.93 ± 10.77ab</td>
</tr>
<tr>
<td>72 h FI (kcal) / BW</td>
<td>0.90 ± 0.05a</td>
<td>0.94 ± 0.04a</td>
<td>0.73 ± 0.04b</td>
<td>1.16 ± 0.04c</td>
</tr>
<tr>
<td>72 h BWΔ (g)</td>
<td>4.77 ± 4.97a</td>
<td>15.88 ± 9.52a</td>
<td>1.60 ± 0.98b</td>
<td>16.39 ± 1.25a</td>
</tr>
<tr>
<td>LBM (g)</td>
<td>211.80 ± 7.86a</td>
<td>228.43 ± 6.65a</td>
<td>158.29 ± 6.65b</td>
<td>161.11 ± 5.86b</td>
</tr>
<tr>
<td>Carcass Fat (g)</td>
<td>48.40 ± 3.97a</td>
<td>64.86 ± 3.36b</td>
<td>67.56 ± 2.96bc</td>
<td>55.29 ± 3.36bc</td>
</tr>
<tr>
<td>Pelt Fat (g)</td>
<td>43.60 ± 3.84a</td>
<td>57.86 ± 3.25b</td>
<td>38.00 ± 3.25a</td>
<td>41.11 ± 2.86a</td>
</tr>
</tbody>
</table>

Body composition

A separate diet and sex effect was observed within subcutaneous (pelt) fat mass (p=0.02, F(6.85, 1) for diet and p<0.00, F(11.34, 1) for sex). Within the pelt data, only males on a HFD had a significantly higher fat mass compared to all other groups (p<0.05) (Table 5.3). No other differences were observed.
Within the carcass data, sex resulted in a significant effect with males displaying an increased amount of lean body mass compared to females (p<0.00; F(79.10, 1)) (Table 5.3). Additionally, HFD resulted in an increase in carcass fat mass (p<0.00; F(17.52, 1). HFD also resulted in a significant increase in carcass fat mass in males and females compared to LFD-fed male rats (p=0.01). Interestingly, HFD did not result in any significant differences between female diet groups in fat mass or lean body mass within the pelt or carcass weights.

Therefore, within this study, the increase in body weight observed after 72 hours of a HFD in females remains unaccounted for but might have been due to an additive effect if entire body adiposity was measured as a whole, compared to measuring adipose stores separately. While rats were randomly assigned to diet, there were no differences in starting weight between diet groups within each sex, suggesting that females were protected from body fat accumulation in this study.

**Spontaneous physical activity**

Spontaneous physical activity was estimated by the distance in meters that each rat moved in the cage over a 24 hour time span. A significant sex effect was observed on all 3 days measured and the entirety of the 72 hours (p=0.02, F(5.72, 1) for day 1; p=0.01, F(8.76, 1) for day 2; p=0.02, F(6.28, 1); p<0.00).

![Distance traveled within the home cage](image)

**Figure 5.1 Distance traveled within the home cage.** As measured by HomeCage Scan by CleverSystems Inc (Reston, VA). Three-month-old male and female Long-Evans rats were fed diets either high in fat or low in fat for 72 hours (LF males: n = 6; HF males: n = 9; LF females: n = 8; HF females: n = 9). Rats were given a 24-hour acclimation period to the behavioral room before the start of the study. Data were collected from the computer program in 24-hour intervals throughout the 72-hour study. Distance traveled represents the distance in meters the rat moved within their home cage during each measurement period. Statistics were performed using 2-way ANOVAs, and individual group differences presented here were measured using Tukey HSD. Means that do not share a common letter indicate statistical difference at P < .05. Abbreviations: LF, low fat; HF, high fat.
F(16.37, 1) for the total value). Male rats fed a HFD moved less than female rats fed both a LF and HFD (p<0.03) on day 2 of the experiment (Figure 5.1). Additionally, this same reduction in distance traveled was seen for the value inclusive of all 3 days. Male rats fed a HFD moved less than females fed either the LFD or HFD (p=0.03 and p=0.04 respectively) (Figure 5.1).

**Lipogenic gene expression**

A significant diet effect was observed in *Fas* gene expression (p<0.00; F(33.86, 1)) (Figure 5.2A). Both males and females fed HFD had reduced *Fas* expression compared to their respective LFD controls (p=0.006 and p=0.014 respectively). Differentially, *Acc1* expression had a significant sex by diet interaction (p=0.02; F(6.43, 1)) (Figure 2A). Females fed a LFD had significantly higher *Acc1* expression compared to all other groups (p<0.02).

Additional genes associated with hepatic lipogenesis were also measured (Figure 5.2A). A significant diet effect was observed in both *Srebp1* (p<0.00; F(12.44, 1)) and *Scd1* (p<0.00; F(23.39, 1)). Both sexes fed
a HFD had decreased $Srebp1$ and $Scd1$ expression compared to the LFD-fed males and females (p<0.03). Lastly, a significant sex by diet interaction was observed in $Dgat1$ expression (p=0.01; F(9.79, 1). Males of both diets and females fed HFD had lower expression of $Dgat1$ compared to female rats fed LFD (p<0.00) and HFD in both sexes reduced expression of $Dgat1$ compared to the LFD-fed males (p<0.00). Interestingly, we saw a significant upregulation of $Ppar\gamma$ expression caused by HFD (p<0.00; F(8.33, 1)). As $Ppar\gamma$ is an upstream regulator of many lipogenic genes, the upregulation of this gene might serve as an early marker of future lipogenic changes.

Lastly, previous work has demonstrated that neuropeptide $Npy$ is capable of influencing and increasing hepatic very low density lipoprotein (VLDL) release [30]. In our study we saw a significant upregulation of $Npy$ expression that was both dependent on HFD (p<0.00; F(11.53, 1)) and the male sex (p<0.00; F(14.09, 1)) (Figure 5.2A). The significant upregulation of hepatic $Npy$ expression in HFD-fed males may also reflect increased serum VLDL content in our study.

Lipolytic gene expression

A sex by diet interaction was seen in $Pnpla2$ expression (p<0.00; F(17.77, 1)) (Figure 5.2B). Females fed a LFD had greater lipolytic gene expression compared to all other groups (P<0.00). The sex differences observed within the low fat controls was lost during high fat feeding. This would suggest reduced metabolism of hepatic triglyceride stores for energy utilization due to HFD, with a greater response in females.
**XBP1 mRNA levels**

Corresponding with the reduced lipogenic expression seen in both sexes fed a HFD for 72 hours, both sexes fed a HFD had reduced spliced Xbp1 mRNA levels compared to LFD-fed males (p<0.0001) (Figure 5.3). This observation also resulted in a significant diet effect (p<0.00; F(27.57, 1)). Total Xbp1 mRNA was also found to have a significant sex effect (p<0.00; F(25.21, 1)). Females fed a HFD had lower total Xbp1 expression compared to both diet groups in males (p<0.002).

**Cell signaling gene expression**

A significant sex by diet interaction was observed in Creb1 (p<0.00; F(13.76, 1)) and Jak2 (p<0.00; F(26.78, 1)) expression (Figure 5.4). Creb1 expression was upregulated in males fed a HFD compared to all other groups (p<0.00). HFD males reduced Jak2 expression compared to their LFD-fed

---

**Figure 5.3 Hepatic Xbp1 gene expression after 72 hours of high-fat feeding.** Three-month-old male and female Long-Evans rats were fed diets either high in fat or low in fat for 72 hours (LF males: n = 6; HF males: n = 9; LF females: n = 5; HF females: n = 9). Xbp1s reflects the spliced, active form of Xbp1, whereas Xbp1t reflects both forms of Xbp1. Data were normalized to GAPDH. Statistics were performed using 2-way ANOVAs, and individual group differences presented here were measured using Tukey HSD. Means that do not share a common letter indicate statistical difference at P < .05. Abbreviations: LF, low fat; HF, high fat.

**Figure 5.4 Hepatic expression of genes involved with major cell signaling pathways.** Three-month-old male and female Long-Evans rats were fed diets either high in fat or low in fat for 72 hours (LF males: n = 6; HF males: n = 9; LF females: n = 5; HF females: n = 9). Data were normalized to GAPDH. Statistics were performed using 2-way ANOVAs, and individual group differences presented here were measured using Tukey HSD. Means that do not share a common letter indicate statistical difference at P < .05. Abbreviations: LF, low fat; HF, high fat.
controls, whereas HFD females increased their expression (P<0.00). Both significant sex (P<0.00; F(33.11, 1)) and diet (P<0.00, F(21.05,1) effects were seen in Mapk expression. Additionally, a significant diet effect was observed in Stat3 expression. In both Mapk and Stat3 expression, HFD resulted in increased expression in both sexes (p<0.01).

Inflammatory gene expression

A significant diet effect was observed in both hepatic Il6 and Tnfa expression (p=0.01, F(8.51, 1) and p<0.00, F(10.74, 1) respectively) (Figure 5.5). In both genes, HFD-fed females had reduced gene expression compared to LFD-fed females (p<0.05). Additionally, HFD-fed females had reduced Tnfa expression compared to LFD-fed males (p<0.01). Females on a HFD also had reduced expression of Socs3 compared to LFD-fed females (p=0.01). Differentially, Socs3 had a significant sex by diet interaction (p=0.02, F(6.10, 1)).

Apoptotic gene expression

Significant diet effects were observed in Anxa5 (p<0.02; F(6.72, 1), Bbc3 (p<0.00; F(11.96, 1)), Casp2 (p<0.00; F(8.14, 1)), and Madh1 (p<0.00; F(8.70, 1)) (Figure 5.6A). HFD resulted in an increase in apoptotic gene expression; however, these were significant only in the females in all

Figure 5.5 Hepatic inflammatory gene expression after 72 hours of high-fat feeding. Three-month-old male and female Long-Evans rats were fed diets either high in fat or low in fat for 72 hours (LF males: n = 6; HF males: n = 9; LF females: n = 8; HF females: n = 9). Data were normalized to GAPDH. Statistics were performed using 2-way ANOVAs, and individual group differences presented here were measured using Tukey HSD. Means that do not share a common letter indicate statistical difference at P < .05. Abbreviations: LF, low fat; HF, high fat.
genes measured (P<0.05). Such results indicate a sex-specific enhancement of pro-apoptotic gene expression when rats are given a short-term exposure to HFDs.

**Cell repair and remodeling expression**

A significant sex by diet interaction was observed in *Bgn* (p<0.00; F(12.82, 1)) and *Sparc* (p<0.01; F(7.72, 1)) (Figure 5.6B). Significant diet effects were seen in the expression of *Fn1* (p<0.02; F(6.15, 1)), *Gata3* (p<0.02; F(6.45, 1)), *Mmp11* (p<0.01; F(7.22, 1)), *Serpinh1* (p<0.01, F(7.40, 1)), and *Vegf* (P<0.00; F(10.35, 1)) (Figure 5.6B). In all genes, HFD feeding resulted in a significant increase in expression in the females (P<0.05), thus suggesting an acute response of greater hepatic recovery when challenged with a HFD.

**Discussion**

In this study female rats fed a HFD for 72 hours were protected from increased adiposity in both the visceral and subcutaneous fat depots. Such protection would be expected because it is commonly observed that females experience slower adipose gains compared to males when fed...
obesogenic diets [29]. This is because female rats consume less energy while expending more energy than similarly treated males, in addition to estradiol-driven actions on adipose, muscle, and hepatic metabolic activity. Interestingly however, HFD-fed females in our study were not protected from increased caloric intake and hyperphagia. The contradiction between caloric intake and body fat observed in our study requires further exploration. Female rats in this study appeared to be more physically active as suggested by the distance traveled over the experiment. This could suggest that the observed protection could be attributed to differences in caloric expenditure or substrate utilization.

As hepatic lipogenesis contributes to adiposity and estrogens have been shown to down-regulate lipogenesis, it was hypothesized that the protection from HF-induced increases in adiposity observed in females was in part due to reductions in lipogenesis [10; 11; 13; 24]. No sex differences emerged in the lipogenic genes measured including Fas, Srebp1c, Dgat1, and Scd1. HFD, regardless of sex, resulted in a down-regulation of most hepatic lipogenic genes compared to LFD controls. These results parallel the findings of a similar study by Ren et al in male C57BL/6J mice [25]. In their study, mice fed a 60% saturated fat diet had significant hepatic lipid accumulation, steatosis, and insulin resistance after 72 hours of the diet. Additionally, the HFD resulted in significant downregulation of hepatic lipogenesis. In combination with our results, the apparent downregulation of lipogenesis after 72 hours of a HFD in both sexes supports a product inhibitory effect of dietary fat on hepatic enzymatic activity. Palmitate is a direct inhibitor of Acc1 function, but additionally downregulates lipogenic gene expression in some models [5; 36]. Therefore, our findings suggest that the switch to a stress-induced upregulation in lipogenesis, a common characteristic of obesity, occurs at a time point beyond 72 hours in both sexes. The upregulation of Pparγ expression in both sexes after 72
84 hours of HFD indicates this transition towards increased lipogenesis occurred shortly after this time period.

*Xbp1* has recently been demonstrated to regulate the transcription of lipogenic genes [19]. In brief, *Xbp1* mRNA splicing, often observed during diet-induced stress, leads to activation of its transcriptional properties. Similar to our results, Ren et al found no significant diet-induced activation of *Xbp1* activity [25]. *Xbp1* was chosen due to its documented activation by saturated fat, which was the significant contributor to dietary fat in our diet; activation of *Xbp1* results in upregulation of hepatic lipogenic enzymes and provides the link between HFDs and increases in lipogenesis [35]. As estradiol has been shown to independently regulate both *Xbp1* and hepatic lipogenesis, a sex difference in *Xbp1* activity was hypothesized in our study [10; 17; 24; 33]. This however was not observed; the amount of active *Xbp1* mRNA reflected the down-regulation of lipogenic expression in both sexes. However, it is important to mention the amount of total *Xbp1* mRNA (inclusive of both spliced and unspliced variants) was significantly decreased in female rats compared to males. While it is difficult to interpret what this could reflect physiologically, to our knowledge we are the first to document a sex difference in total *Xbp1* expression in the liver.

Recent studies have demonstrated that HFDs consumed for 72 hours promote the development of systemic and central insulin resistance [3; 7; 20; 22; 25]. Saturated fatty acids, in particular palmitate, induce insulin resistance by activation of inflammatory signaling within cells [8]. *Il6, Socs3,* and *Tnfa* all have been associated with induction of hepatic insulin resistance [26; 28]. In addition, liver specific inhibition of *Socs3* resulted in a suppression of hepatic insulin resistance in several models [26; 32]. Combined with this knowledge, the HF dietary-induced reduction in hepatic inflammatory genes in our study suggests a potential
retention of insulin sensitivity. The HFD in our study did not cause inflammatory gene expression in the liver, therefore greatly reducing the potential for cytokine-induced insulin resistance. This finding is conflicting with the conclusion from a similar study, which found that hepatic insulin resistance at 72 hours was dependent on Kupffer cell activation and inflammatory signaling in male mice [18]. One major limitation to our study is the lack of investigation of insulin resistance in females, which has yet to be determined in this model. While we are the first to report data in females in short-term exposures to HF diet, future research needs to investigate differences in insulin signaling between the sexes to help address the above questions.

Previous 72 hour HFD studies found that both serum cytokines and hepatic inflammatory expression is increased, in congruence with insulin resistance in males [18; 20]. These observations directly oppose what we observed, however the difference in inflammation-associated gene expression may be due to differences in the amount of dietary fat between the studies (60% fat compared to 40% fat). The diet used in this study has demonstrated pro-inflammatory properties during longer intake studies [15]. This diet, being lower in fat compared to other 72 hour studies, might not have been as stressful and thus did not promote inflammatory gene expression. Furthermore, it is also possible that the differences in overall nutrient composition between our HF diet and others used in the literature may also be a contributing factor. It is important to note the increased Stat3 expression caused by HFD in our study. As Stat3 is an upstream regulator of the inflammatory cytokines involved in insulin resistance, a progression towards the pro-inflammatory state could have been occurring and was just not observed during our time period with our chosen diet [16].

Lastly, we report a novel finding that 72 hours of HFD results in increased expression of genes related to apoptosis, cell repair, and cell remodeling. Interestingly, HFD upregulated such
genes to a far greater extent in females than in male rats. We did not measure serum alanine transaminase in our study, which would further support the sexual dimorphic response seen in the liver and thus is a limitation to our work. However, acute apoptotic gene expression can be advantageous in regards to preventing disease and normally occurs within the liver without induction of pro-inflammatory cytokines [12]. As this study is an early injury design, the increase in apoptotic gene expression seen in females can be considered a protective mechanism. The pro-apoptotic effects of estrogen during acute traumas have been previously documented and reviewed in the literature [27; 31]. As an acute upregulation of apoptotic genes are important during transient injuries, the sex difference observed in our study might serve as a novel pathway by which females are protected from steatosis and hepatocarcinomas [12; 23].

To our knowledge we are the first to demonstrate that both sexes equally down-regulate hepatic lipogenic gene expression after 72 hours of a HFD. This finding opposes what our original hypothesis stated. However, we did measure a greater response caused by the HFD in genes related to inflammation, apoptosis, cell repair, and cell remodeling in female rats. This may suggest a novel mechanism behind the observed sex differences in liver disease. Lastly, both sexes consume the same amount of energy over this time span, ameliorating the sex-difference common when rats are fed standard chow. Interestingly, female rats were protected from increases in adiposity in both the visceral and subcutaneous fat deposits. Because this protection was not due to differences in caloric intake or hepatic lipogenesis, why female rats are protected from increased adiposity after 72 hours of a HFD remains to be determined.
Acknowledgements

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References


CHAPTER 6

A DIETARY PHYTOCHEMICAL BLEND PREVENTS LIVER DAMAGE ASSOCIATED WITH ADIPOSE TISSUE MOBILIZATION IN OVARIECTOMIZED RATS

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Abstract

Objective: Menopausal reduction in estrogen causes increased adipose accumulation, leading many to turn to dietary supplements to prevent and treat such changes. Enhanced adipose mobilization stimulated by some supplements can increase the risk of non-alcoholic fatty liver disease (NAFLD). Cytoprotective and anti-obesity compounds may prevent the lipotoxicity associated with mobilization.

Methods: A phytochemical blend was tested in aged, ovariectomized rats. Rats were given the AIN-93M basal diet or a diet containing varying doses of phytochemicals with 2.4 IU/g vitamin D (diet 1: 1000 mg/kg Genistein (G); diet 2: 500 mg/kg (G), 200 mg/kg Resveratrol (R), and 1000 mg/kg Quercetin (Q); diet 3: 1000 mg/kg (G), 400 mg/kg (R), and 2000 mg/kg (Q)).

Results: Serum free fatty acids and hepatic triglycerides were elevated with diets 2 and 3. Despite this increase, the phytochemical blends did not increase apoptotic, cell repair, or remodeling gene expression. The highest phytochemical dose prevented increases in serum alanine aminotransferase.

Conclusion: Adverse hepatic effects secondary to ovariectomy were mitigated through the inclusion of a dietary phytochemical blend in aged ovariectomized rats. The use of such compounds may not only help with weight management and disease risk in menopausal women, but may also prevent the lipotoxicity in NAFLD.

Introduction

The menopausal loss of 17B-estradiol (E2) reverses the sex-specific protection that females have from the development of many diseases including cardiovascular and hepatic diseases (1). Furthermore, the reduction of E2 during this time causes weight gain and increased visceral fat...
mass (2, 3). With the reluctance over hormone replacement therapy use, mid-life weight gain may lead many to over-the-counter weight loss supplements to manage changes in body composition.

While the efficacy of weight loss supplements are controversial, animal studies of some popular compounds have pinpointed the liver as a site of supplement-induced tissue damage. Compounds such as conjugated linoleic acid (4) that have significant lipolytic effects in adipocytes cause increased lipid mobilization to the liver in addition to increased hepatic lipogenesis, insulin resistance, and reduced fatty acid oxidation (5, 6, 7). The fat deposition in the liver which is attributed to such compounds promotes a lipotoxic effect, causing downstream inflammatory signaling, apoptosis, and development of fibrosis.

Estrogenic compounds have proven to be beneficial to hepatic health. Phytoestrogens including resveratrol and genistein reduce hepatic lipogenesis, inflammation, and apoptosis (8, 9). Furthermore, such compounds appear to promote lipolysis and protection from high fat diet-induced weight gain in vivo. However, as singular compounds, human equivalent doses are too high and thus too difficult to achieve for effectiveness within the human population (10). Studies from our lab have identified a lower-dose, synergistic combination of phytoestrogens that have proven to be anti-adipogenic (11, 12). The impact of this combination on hepatic lipid and health has yet to be investigated.

For this study we investigated varying doses of a synergistic phytochemical blend of resveratrol, genistein, and quercetin on overall hepatic health in ovariectomized (OVX) rats. OVX causes an increase in hepatic lipid deposition and a trend towards steatosis due to the loss of E2, closely mimicking what is observed during menopause in females (13). Thus, we hypothesized that OVX would cause increased lipogenesis and lipid deposition in the liver. It
was also hypothesized that phytochemical treatment would reverse any mal-effects that were produced by OVX in the rats, and protect the liver from any lipotoxic effects associated with lipid mobilization due to treatment.

**Methods**

*Animals and housing*

Retired breeder Fischer 344 rats (n=60) were purchased from the National Institute of Aging colony at Taconic and Charles River Laboratories (Germantown, NY and Wilmington, MA respectively). The study was carried out in two separate blocks of 30 rats each. Prior to sham or ovariectomy surgery, rats were randomly assigned to treatment groups. Following 1 week of recovery, rats were fed either the basal diet (AIN-93M) or the modified diet containing 2.4 IU/g vitamin D with phytochemical compounds that were mixed in (Table 6.1). Rats had free access to tap water and the powdered diet were available ad libitum. Daily food intake and weekly body weights were measured. At the end of 16 weeks, rats were fasted for 2 hours and euthanized by decapitation under 2.5% isoflurane. The study was approved by the University of Georgia Institutional Animal Care and Use Committee.

**Table 6.1 Diet Composition.** Rats were fed the AIN-93M diet from TestDiets. Compounds were purchased from Spectrum Chemicals (Gardena, CA). Phytochemicals were mixed into the powdered diets at the necessary doses. Abbreviations: ovariectomized (OVX).

<table>
<thead>
<tr>
<th>Energy (%)</th>
<th>Sham</th>
<th>OVX</th>
<th>OVX + genistein</th>
<th>OVX + low dose blend</th>
<th>OVX + high dose blend</th>
</tr>
</thead>
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<tr>
<td>Protein</td>
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<tr>
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<td>9.7</td>
<td>9.7</td>
<td>9.7</td>
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</tr>
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<td>Kcal/g</td>
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<table>
<thead>
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<th>Compound</th>
<th>Sham</th>
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<th>OVX + genistein</th>
<th>OVX + low dose blend</th>
<th>OVX + high dose blend</th>
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</thead>
<tbody>
<tr>
<td>Vitamin D₃ (IU/kg of diet)</td>
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<td>1000</td>
<td>2400</td>
<td>2400</td>
<td>2400</td>
</tr>
<tr>
<td>Genistein (mg/kg of diet)</td>
<td>0</td>
<td>0</td>
<td>1000</td>
<td>500</td>
<td>1000</td>
</tr>
<tr>
<td>Resveratrol (mg/kg of diet)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>400</td>
</tr>
</tbody>
</table>
**Hepatic gene expression**

Tissue samples were snap frozen in liquid nitrogen immediately upon dissection. RNA was isolated using a Qiagen MiniKit (Valencia, CA) according to the manufacturer’s instructions and the final RNA concentration was determined with the NanoDrop 8000 spectrophotometer (Wilmington, DE). Two micrograms of RNA was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription kit by Applied Biosystems (Grand Island, NY). Quantitative PCR was run to determine differences in gene expression using Applied Biosystems TaqMan master mix and pre-designed primers on the 7500 Prism system (Applied Biosystems, Grand Island, NY) (Table 6.2).

**Table 6.2 Primers used for real-time PCR.** Fischer 344 retired breeders were ovariectomized and fed a synergistic phytochemical diet for 16 weeks. Liver samples were removed and snap frozen in liquid nitrogen for quantitative PCR analysis. Pre-designed TaqMan primers were purchased from Applied Biosystems.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Gene symbol</th>
<th>Gene Name</th>
<th>Context sequence</th>
</tr>
</thead>
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<tr>
<td>Endogenous control</td>
<td>ActB</td>
<td>Beta actin</td>
<td>Rn00667869_m1</td>
</tr>
<tr>
<td></td>
<td>18S</td>
<td>45S pre-ribosomal RNA</td>
<td>Rn00573474_m1</td>
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<tr>
<td>Lipogenic</td>
<td>ACACA</td>
<td>Acetyl Co-A carboxylase alpha (ACC1)</td>
<td>Rn00584879_m1</td>
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<tr>
<td></td>
<td>DGAT1</td>
<td>Diglyceride acyltransferase 1</td>
<td>Rn00573474_m1</td>
</tr>
<tr>
<td></td>
<td>FASN</td>
<td>Fatty acid synthase</td>
<td>Rn00573474_m1</td>
</tr>
<tr>
<td></td>
<td>SCD1</td>
<td>Sterol Co-A desaturase</td>
<td>Rn00594894_g1</td>
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<tr>
<td></td>
<td>SREBF1</td>
<td>Sterol regulatory element-binding protein 1</td>
<td>Rn01495769_m1</td>
</tr>
<tr>
<td></td>
<td>XBP1</td>
<td>X-box binding protein 1</td>
<td>Rn01443523_m1</td>
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<td>Apoptosis</td>
<td>ANXA5</td>
<td>Annexin 5</td>
<td>Rn00565571_m1</td>
</tr>
<tr>
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<td>BBC3</td>
<td>Bcl-2 binding component 3</td>
<td>Rn00597992_m1</td>
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<tr>
<td></td>
<td>CASP2</td>
<td>Caspase 2</td>
<td>Rn00574684_m1</td>
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<tr>
<td></td>
<td>MADH1</td>
<td>Mothers against decepentaplegic homolog 1</td>
<td>Rn00565555_m1</td>
</tr>
<tr>
<td>Cell repair and remodeling</td>
<td>BGN</td>
<td>Biglycan</td>
<td>Rn01529736_m1</td>
</tr>
<tr>
<td></td>
<td>FN1</td>
<td>Fibronectin</td>
<td>Rn00569575_m1</td>
</tr>
<tr>
<td></td>
<td>MMP11</td>
<td>Matrix metallopeptidase 11</td>
<td>Rn00564319_m1</td>
</tr>
<tr>
<td></td>
<td>SERPINH1</td>
<td>Serpin peptidase inhibitor clade H member 1</td>
<td>Rn00567777_m1</td>
</tr>
<tr>
<td></td>
<td>SPARC</td>
<td>Osteonectin</td>
<td>Rn01470624_m1</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
<td>Rn01511604_m1</td>
</tr>
</tbody>
</table>
**X-box binding protein 1 (XBP1) splicing assay**

The original protocol to quantify spliced XBP1 mRNA in pre-designed primers was developed by Hirota et al (14). To create double stranded DNA, cDNA, PCR master mix, and the Applied Biosystems XBP1 primer was combined and ran under 2 cycles of the PCR reaction. Samples were then removed and 0.75 U of PstI (Promega, Fitchburg, WI) was added to each sample for 1 hour. Quantitative PCR on the samples was then run under standard operating conditions on the 7500 Prism system (Applied Biosystems, Grand Island, NY).

**Hepatic and adipose lipid accumulation**

Lipid was isolated from tissue samples using the Folch Method previously described (15). In brief, tissue was homogenized in a 2:1 solution of chloroform and methanol and left to incubate overnight at 4°C. The next day the sample was centrifuged at 2,000 rpm for 15 minutes and the supernatant was kept and washed with 1% NaCl. This mixture was vortexed briefly before being centrifuged again at 2,000 rpm for 10 minutes. The top layer was removed and discarded, and the bottom layer was dehydrated. Lipid weight was determined by obtaining the weight of the empty microcentrifuge tube subtracted from the dry lipid sample. To measure hepatic triglycerides, the dried lipid sample was resuspended in 1% Triton X-100. The sample was then loaded in duplicate onto a 96-well plate along with the colorimetric Triglyceride (TAG) Reagent (Pointe Scientific, Canton, MI). Absorbance was read using the FlexStation 3 (Molecular Devices, Sunnyvale, CA) and final TAG concentration was determined using manufacturer’s instructions.
Histology

Hepatic samples were frozen on dry ice at the time of sacrifice and later stored at -80°C until use. Sections of each liver sample were made with a cryostat-microtome set at 6 µm. The livers were stained using Oil Red O with hematoxylin as a counter nuclear stain. H&E stains were performed at UGA College of Veterinary Medicine Pathology Laboratory. Slides were imaged at 100x magnification.

Total protein isolation

Protein from frozen tissue was homogenized and isolated using RIPA Buffer supplemented with 10% protease inhibitors (Sigma-Aldrich, St. Louis, MO). Total protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Serum assays

Trunk blood was collected and allowed to clot for 30 minutes at room temperature. Blood was centrifuged at 2,000 x g for 15 minutes at 4°C to separate serum and stored at -80°C. Serum activity of Alanine Aminotransferase (ALT) was measured using spectrophotometric clinical assays kit (Pointe Scientific, Canton, MI). Serum free fatty acid (FFA) was measured by ELISA (Cayman Chemical, Ann Arbor, MI) in accordance with the manufacturer’s instructions using the FlexStation 3 (Molecular Devices, Sunnyvale, CA).

Statistics

Statistics were performed using STATISTICA software (version 7.0; Tulsa, OK, USA). One-way analysis of variance was used to determine significant treatment effects. Significant
differences among treatment groups was measured using Fisher’s Least Significant Difference test at p<0.05.

**Results**

*Body weight, food intake, and tissue weights*

There were no differences in initial body weight or final body weight between groups (Table 6.3). A trending treatment effect was measured in average daily food intake (p<0.06). No treatment effects were measured in the retroperitoneal or inguinal adipose depot, gastrocnemius, soleus, or total liver weight. Lastly, a treatment effect was measured in uterus weight. Ovariectomy resulted in a reduction in uteri weight that was not reversed with any of the phytochemical diets (p<0.00).

**Table 6.3 Rat characteristics.** Data are presented as mean ± SEM values. Statistics were performed using ANOVA and Fisher LSD was used for post-hoc analysis. Means that do not share a common letter are statistically significant at p<0.05. Abbreviations: ovariectomized (OVX), brown adipose tissue (BAT), inguinal (I), retroperitoneal (RP).

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>OVX</th>
<th>OVX + genistein</th>
<th>OVX + low dose blend</th>
<th>OVX + high dose blend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>248.93</td>
<td>242.42</td>
<td>246.13 ± 3.83</td>
<td>242.83 ± 5.17</td>
<td>250.50 ± 3.95</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>311.71</td>
<td>315.44</td>
<td>316.54 ± 5.50</td>
<td>317.79 ± 4.21</td>
<td>319.31 ± 5.38</td>
</tr>
<tr>
<td>Daily food intake (g)</td>
<td>13.64</td>
<td>12.96</td>
<td>12.85 ± 0.17b</td>
<td>13.23 ± 0.19ab</td>
<td>12.89 ± 0.18b</td>
</tr>
<tr>
<td>Adipose tissues</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAT (mg)</td>
<td>904.83</td>
<td>819.76</td>
<td>818.25 ± 0.03b</td>
<td>812.00 ± 0.02b</td>
<td>763.00 ± 0.04b</td>
</tr>
<tr>
<td>RP (g)</td>
<td>11.08</td>
<td>11.65</td>
<td>12.34 ± 0.50</td>
<td>11.46 ± 0.45</td>
<td>11.14 ± 0.32</td>
</tr>
<tr>
<td>I (g)</td>
<td>13.93</td>
<td>16.18</td>
<td>15.45 ± 0.57</td>
<td>15.66 ± 0.59</td>
<td>15.37 ± 0.74</td>
</tr>
<tr>
<td>I + RP (g)</td>
<td>25.01</td>
<td>27.83</td>
<td>27.78 ± 0.83</td>
<td>27.13 ± 0.88</td>
<td>26.51 ± 0.93</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Gastrocnemius (g)</td>
<td>2.45</td>
<td>2.49</td>
<td>2.46 ± 0.03</td>
<td>2.48 ± 0.03</td>
<td>2.51 ± 0.04</td>
</tr>
<tr>
<td>Soleus (g)</td>
<td>0.16</td>
<td>0.14</td>
<td>0.15 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.16 ± 0.01</td>
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<tr>
<td>Uteri (mg)</td>
<td>0.48</td>
<td>0.20</td>
<td>0.18 ± 0.01b</td>
<td>0.17 ± 0.01b</td>
<td>0.17 ± 0.01b</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>9.71</td>
<td>8.65</td>
<td>8.95 ± 0.25</td>
<td>9.41 ± 0.35</td>
<td>9.15 ± 0.27</td>
</tr>
</tbody>
</table>
Hepatic steatosis

For both sample lipid and the adjusted lipid measurement, OVX resulted in an increase in extracted lipid weight that was not reversed by phytochemical treatment (p<0.02) (Figure 6.1B). Oil Red O staining confirmed increases in hepatic lipid content in all OVX animals compared to sham-treated rats (Figure 6.1A). After resuspension of extracted lipid, a treatment effect was measured in hepatic TAG content. Treatment with the phytochemical blends resulted in an increase in hepatic TAG (p<0.02) (Figure 6.1C).

Figure 6.1 Hepatic steatosis. Representative liver sections were obtained from ovariectomized retired breeders after 16 weeks of phytochemical feeding and stained with Oil Red O (top) and H&E (bottom) (A). An ovariectomy effect on increasing hepatic lipids were observed, however low dose and high dose phytochemical treatment was significantly increased compared to all other groups. Magnification 100x. Hepatic lipid weight was determined by finding the difference between the final lipid extract in a microcentrifuge tube and the empty tube. Lipid weights were adjusted by hepatic protein content (B). Lipid was resuspended in Triton-X prior to colorimetric assay of triglycerides (C). Statistics were performed using ANOVA and Fisher LSD was used for post-hoc analysis. Means that do not share a common letter are statistically significant at p<0.05. Abbreviations: ovariectomized (OVX), triglycerides (TAG).
Retroperitoneal lipid content

After adjustment to adipose weight, ovariectomy increased retroperitoneal lipid weight which was reversed by all phytochemical treatments (p<0.03) (Figure 6.2).

![Retroperitoneal lipid content](image)

**Figure 6.2 Retroperitoneal lipid content.** Lipid was isolated from frozen tissue sample using a chloroform:methanol extraction. Lipid weight was determined by finding the difference between the final lipid extract in a microcentrifuge tube and the empty tube. Lipid weights were adjusted to the adipose sample. Data are presented as mean ± SEM values. Statistics were performed using ANOVA and Fisher LSD was used for post-hoc analysis.Means that do not share a common letter are statistically significant at p<0.05. Abbreviations: ovariectomized (OVX).

Lipogenic gene expression

Diglyceride acyltransferase (DGAT1) expression was reduced by the genistein-only treatment compared to sham controls (p<0.01) (Figure 6.3B). OVX rats had a higher expression of fatty acid synthase (FASN) compared to sham rats and the low dose phytochemical blend treatment (p<0.02) (Figure 6.3C). Sterol Co-A desaturase (SCD1) expression was higher in OVX rats compared to sham control rats, which was reversed with the low dose and high dose phytochemical blends (p<0.04) (Figure 6.3D). Rats given the genistein-only and the high dose phytochemical blend had reduced expression of total XBPI compared to sham rats (p<0.04) (Figure 6.3F). Furthermore, the genistein-only group had reduced XBPI expression compared to the low dose phytochemical blend (p<0.03). No differences were seen in acetyl Co-A carboxylase alpha (ACACA) or sterol regulatory element-binding protein 1 (SREBF1).
**Hepatic apoptotic gene expression**

No treatment effect was measured in annexin 5 (ANXA5) or BCL-2 binding component 3 (BBC3) expression (data not shown). OVX resulted in an increase in caspase 2 (CASP2) expression (p<0.04), which was reversed with all phytochemical treatments (p<0.01) (Figure 6.4A). All phytochemical treatments had reduced mothers against decepentaplegic homolog 1 (MADH1) expression compared to OVX rats (p<0.03) (Figure 6.4B).

**Hepatic cell repair and remodeling gene expression**

No treatment effect was measured in serpin peptidase inhibitor clade H member 1 (SERPINH1) expression (Figure 6.4F). The increase in biglycan (BGN) expression caused by OVX was reversed by phytochemicals (p<0.03) (Figure 6.4C). The genistein-only treatment and low dose
The high dose phytochemical treatment caused an increase in matrix metallopeptidase 11 (MMP11) expression compared to all other groups other than the genistein-only treatment (p<0.03) (Figure 6.4E). Osteonectin (SPARC) expression had a treatment effect, with a similar pattern of an OVX-induced increase that the OVX-induced increase in SPARC expression was also reversed with phytochemical treatments (p<0.04) (Figure 6.4G). Lastly, both phytochemical blend treatments reduced vascular endothelial growth factor (VEGF) expression compared to the sham and OVX controls (p<0.03) (Figure 6.4H).

**Figure 6.4 Hepatic apoptotic, cell repair, and remodeling gene expression.** Data were normalized to Beta Actin and ovariectomized rats fed the control diet. Statistics were performed using ANOVA and Fisher LSD was used for post-hoc analysis. Means that do not share a common letter are statistically significant at p<0.05. Abbreviations: ovariectomized (OVX), caspase 2 (CASP2), mother against decepentaplegic homolog 1 (MADH1), biglycan (BGN), fibronectin (FN1), matrix metallopeptidase 11 (MMP11), osteonectin (SPARC), vascular endothelial growth factor (VEGF).
Serum assays

Sham controls had a higher serum TAG level compared to all other groups (p<0.03) (Figure 6.5A). Treatment with both phytochemical blends increased serum FFA compared to all other groups (p<0.02) (Figure 6.5B). OVX resulted in an increase in ALT compared to intact rats (p<0.03) (Figure 6.5C). Rats on the low dose phytochemical blend had greater ALT compared to sham (p<0.00). High dose phytochemical treatment reversed any increases caused by OVX (p<0.02) and treatment (p<0.00).

Discussion

In this study a comparatively low dose synergistic phytochemical blend did not cause adverse effects in the liver. Increased serum FFA are indicative of enhanced lipid mobilization and has been documented in singular phytochemical studies with genisteen (16). We were able to demonstrate that the low and high dose treatments induced a similar increase in FFA compared to controls. Lipid extraction of the retroperitoneal adipose depot found a significantly increased visceral lipid weight with OVX that was reversed with phytochemical treatment. This finding, in addition to the previous work from our lab in 3T3-L1 cells, supports enhanced lipid mobilization with our phytochemical blend (11).
In addition to the phytochemical-induced influx of lipids to the liver, OVX resulted in a reduced efflux of lipids from the liver. OVX results in increased hepatic lipid accumulation in part due to reduced lipid release from the liver to peripheral tissues (13, 17). Furthermore, E2 increases hepatic TAG secretion in normal but not OVX rats (13, 18). Therefore, our finding that the estrogenic compounds used in our study failed to increase serum TAG and hepatic lipid efflux is not surprising. The increased lipids to and decreased lipids away from the liver, resulted in a significantly higher hepatic TAG and Oil Red O staining in both the low and high dose phytochemical groups compared to controls.

While the majority of hepatic lipid content is adipose-derived, both NAFLD and OVX can increase the percentage of hepatic lipid contributed from de novo lipogenesis (19, 20, 21). Hepatic lipogenic enzymes were measured in our study and overall the data was inconclusive. OVX failed to increase the mRNA of the gene for ACC1 but did for FAS. Congruent with previous literature (20), OVX caused a rise in SCD1 expression. SCD1 has been shown to be an initiating factor in hepatic inflammation, oxidative stress, and insulin resistance (20, 22, 23). Interestingly, OVX-induced increase in SCD1 expression was reversed in both phytochemical blend groups. As hepatic insulin resistance is a contributing factor of NAFLD, SCD1 may be an intriguing future target for natural compound research.

Inflammation being an important promoter of lipotoxicity-induced hepatic damage, compounds with anti-inflammatory properties may be able to prevent the second hit of NAFLD from progressing (24, 25). OVX, as expected, resulted in an increase in pro-apoptotic expression of CASP2 and MADH1 (p<0.06). Microarray and gene enrichment analysis of a similar study involving OVX and singular phytochemical doses measured increased apoptosis, reversed by genistein treatment (26). Genes found to be impacted by genistein included CASP2 but did not
include MADH1, also referred to as SMAD1. MADH1/SMAD1 has been implicated as a contributing pro-inflammatory, pro-apoptotic, and pro-fibrotic signaling protein (26). In our study, phytochemical treatment prevented increases in MADH1 expression caused by OVX. Additionally, other genes related to cell remodeling were also reduced with the phytochemical blend.

Serum ALT was measured to clarify overall hepatocellular health after treatment. As expected ALT increased with OVX. The low dose did not significantly increase serum ALT levels compared to the OVX group. However, the high dose phytochemical blend completely reversed the OVX induced increase in serum ALT, suggesting an improved hepatic function comparable to sham rats.

Despite increased lipid mobilization caused by our treatment, the higher dose blend that was used in this study prevented hepatocellular damage associated with loss of estrogen. Lastly, it is important to restate that the dosages used in our study have demonstrated synergistic properties and thus were designed to be used at a far lower concentration than what is used in singular compound studies (11). With this lowered dose we were able to promote lipid mobilization to the liver which often causes lipotoxicity, however due to the anti-apoptotic properties of the selected phytochemicals we were able to prevent liver damage. It is therefore possible that this phytochemical blend may be used safely and effectively to manage overall changes to body composition caused by menopause.

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References


CHAPTER 7

SUMMARY

The data provided in this dissertation follows females' risk of non-alcoholic fatty liver disease (NAFLD) across the lifespan from birth to menopause in a variety of conditions. Epidemiological studies have demonstrated that females are at a lower risk of NAFLD and other liver diseases compared to their male counterparts at various different time points throughout life including pre-menopausal adulthood and interestingly childhood. While many of the female sex-specific protections from cardio-metabolic diseases have been attributed to the hormone estradiol, the early life sex differences in fatty liver would suggest an inherent epigenetic sex difference at birth.

Chapter 4 was the second liver-based paper that investigated differences between sexes in offspring of diet-induced obese dams. Furthermore, we were the first to investigate insulin signaling cascades in this model. Our data indicated that there is a significant difference in how the sexes respond to maternal obesity in the offspring. Females from DIO dams had increased expression of insulin receptor substrate 1, whereas the males had reduced expression of the insulin receptor. While in both sexes hepatic triglyceride levels were increased when their mothers were obese, the differences in hepatic insulin signaling may lead to a divergent risk of NAFLD during childhood and even later in life. This exciting finding is supported by preliminary data discussed in other papers and provides the first explanation of the observed sex differences in childhood NAFLD. Furthermore, the metabolic differences between the sexes that
was measured in this study provides strong evidence that researchers must separate the sexes in similar research.

In Chapter 5 an acute high fat diet induced injury model was used to study the metabolic and cellular repair expression between male and female rats. Both sexes responded similarly in regards to hepatic metabolic signaling, although females failed to replicate the high fat diet-induced increases in adiposity that was measured in males. The major sexual dimorphism in gene expression was within the apoptosis, repair, and remodeling pathways. A very large increase in these related genes was seen in the females fed a high fat diet, which was not observed in the male group. While this would be damaging to the liver over the long term, this rapid response in the liver of females may very well be protective. Estradiol has been shown to be both pro-inflammatory and anti-inflammatory depending on the acute or chronic pathogen exposure. Females have a heightened response to acute infections compared to males, which is actually reversed in chronic diseases. Therefore the unanticipated response in this study may be protective and contribute to the sex differences observed in hepatic disease.

Lastly, we investigated the ability of a protective phytoestrogen blend to reverse some of the symptoms of menopause, including NAFLD, in the rodent ovariectomy model. The compounds (resveratrol, quercetin, and genistein) were chosen due to synergistic anti-adipogenic effects that have been demonstrated in our laboratory previously. Like most anti-obesity compounds that have specific effects on the adipocyte, the blend used in our study induced lipid mobilization to the liver. However, unlike other compounds, this blend is cellular protective and was found to prevent liver damage and steatohepatitis when hepatic apoptotic gene expression and serum alanine amino transferase was measured. While we were able to replicate the increased hepatic lipid that occurs after ovariectomy, we were actually able to prevent the
damage caused by both ovariectomy and lipid mobilization. Therefore, this phytochemical blend may prove to be safe and effective for use in post-menopausal women to help reduce the burden of estrogen deficiency.

In summary, the data in this dissertation provides the evidence that females are protected from the development of NAFLD by three potential mechanisms: (1) an inherent difference in endoplasmic reticulum stress and insulin sensitivity at birth that is most likely epigenetically regulated, (2) a rapid and heightened response to acute hepatic injuries including short exposures to high fat diets, and (3) the anti-inflammatory and cytoprotective effects of estradiol and its mimetics.
CHAPTER 8

FUTURE DIRECTIONS

**Single cell type based assays**

Further studies are necessary to investigate the mechanisms proposed in this dissertation. To confirm the role of epigenetic modifications related to sex at birth, it is necessary that specific methods be developed. Current research in the field of epigenetics has pinpointed the need for cell type specific assays to be able to measure epigenomic modifications.

The liver is a highly dynamic and responsive tissue that contains multiple cell types beyond the hepatocyte including the macrophage-like Kuppfer cells, vascular cells, and stem cells. Currently scientific methods for genomic isolation involves homogenization of whole tissue, and thus the isolated RNA and DNA from the tissue contains all of the cell types within it. When we analyze differences within this genomic salad, it has been documented that the specific differences within in each cell type can cancel each other out. For example, if gene expression is repressed in the hepatocyte but upregulated within the Kuppfer cell, the overall effect will be no change within the tissue. This methodological problem may have severely impacted any study involved with epigenomic data.

Development of techniques to separate the cell types found within a tissue is the exciting future of both genomic and epigenetic work. With collaborations between the Baile and Meagher laboratories, we hope to develop the necessary tools and protocols to be able to isolate hepatocyte nuclei from whole tissue in the near future, thus allowing for the role of epigenetics to be studied in the noted metabolic sex differences early in life.
Mechanistic studies in vitro

It has been well established that estradiol is protective against NAFLD. Surprisingly, only one mechanistic-based study has been published which highlighted the liver X receptor as a modulatory pathway by which estradiol may be working. Even more surprisingly, no studies exist in vitro. A further aim in our lab is to investigate the XBP1-estradiol link within the human HepG2 cell model. Some preliminary data related to this future aim is discussed below.

Methods

Human HepG2 cells were purchased from ATCC and maintained in RPMI 1640 media supplemented with 10% FBS. Experiments were performed using a phenol-red free medium with charcoal-stripped FBS purchased from Life Technologies to eliminate any estrogens within the growth medium. Cells were grown to 60% confluency, at which point the media was replaced with media containing compounds including palmitate (lipogenic inducer), rapamycin (endoplasmic reticulum stress inhibitor), estradiol, and ICI 182,780 (estrogen receptor alpha inhibitor). Cells were grown within the treatment media for an additional 24 hours.

Dose response curves

Palmitate, being solid at room temperature, was conjugated to free fatty acid free-BSA prior to use. Based on previous literature, we picked varying doses from 0-1 uM. Cells were treated for 24 hours and then assayed using AdipoRed reagent to measure lipid accumulation and Cell Titer
Blue to measure viable cells. A curve was not established in doses lower than 1 uM (Figure 8.1). However, palmitate treatment did significantly increase lipid accumulation in viable cells. The effect on BSA was also measured and was found to also increase lipid accumulation at doses higher than 0.50 mM. Therefore, it was decided that the final dose for palmitate for this study would be 0.50 mM. A rapamycin curve was then developed to confirm that lipid accumulation could be reversed with an endoplasmic reticulum stress inhibitor. Using the selected palmitate dose, a final rapamycin dose of 10 nM was found to enhance cell viability and reduce lipid accumulation (Figure 8.2). Thus, reversing the effects of palmitate treatment in HepG2 cells.

Figure 8.1 Palmitate dose curve. Effects of 24 hours of palmitate treatment on lipid accumulation in viable HepG2 cells.

Figure 8.2 Rapamycin dose curve. Effects of 24 hours of rapamycin treatment on lipid accumulation in viable HepG2 cells treated subsequently with 0.50 mM palmitate.
An estradiol curved was established. Both AdipoRed and Cell Titer Blue were run to determine a non-toxic and effective dose of estradiol and its inhibitor. Powdered estradiol was dissolved in ethanol and cells were treated with varying doses for 24 hours. A control for the ethanol solvent indicated no effect on either lipid accumulation or cell viability. Estradiol treatment increased cell viability up to 1 uM dose and reduced lipid accumulation in viable cells most effectively in the 50 nM dose (Figure 8.3). Therefore, 50 nM estradiol was chosen. In summary, for this study we identified the following doses for continuation of this investigation: 0.50 mM palmitate, 10 nM rapamyacin, and 50 nM estradiol.

Translation

The next phase of this project is to complete dose response curves including the inhibitor of estrogen receptor alpha, ICI 182, 780, and various endoplasmic reticulum stress activators. By using various combinations of endoplasmic reticulum stress activators and repressors, estradiol and its inhibitor, and palmitate we will determine a novel mechanism by which estradiol may be regulating hepatic lipogenesis and thus non-alcoholic fatty liver disease.