MILLER, COLETTE N., M.S. Estradiol Reduces Inflammation in Rats Fed a High-fat Diet. (2010)
Directed by Dr. Lynda M. Brown. 98 pp.

Previous research has shown that leptin and insulin resistance can occur after rats are fed a high-fat (HF) diet for 72 hours. Because leptin and insulin resistance can be a result of HF diet-induced inflammation, the purpose of this research was to determine if inflammatory gene expression occurs after 72 hours of a HF diet. Additionally, since estrogen (E2) is anti-inflammatory, the extent of which intact females and ovariectomized (OVX) express inflammatory cytokines after 72 hours of a HF diet was also determined.

Intact females in proestrus had reduced hypothalamic inflammatory gene expression of TNFα, whereas males had increased hypothalamic expression of SOCS3 after 72 hours of a HF diet. Within the liver, females in proestrus had reduced expression of all genes measured in addition to reduced XBP1 mRNA after a HF diet. However, no such reductions were observed within males.

To determine if these reductions in inflammatory gene expression was due to the increased circulating E2 seen during proestrus, E2 was reintroduced in an OVX model. After 24 hours of a HF diet, E2 treatment prevented increases in hypothalamic SOCS3 expression. However, this protection was attenuated at 72 hours and no other treatment-induced changes were observed.

ESTRADIOL REDUCES INFLAMMATION IN RATS FED A HIGH-FAT DIET

by

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A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2010

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APPROVAL PAGE

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ACKNOWLEDGEMENTS

I would like to thank Dr. Lynda Brown and my committee members, Dr. Keith Erikson and Dr. Ron Morrison for their guidance. Additionally, I would like to thank Paula Cooney for her technical assistance throughout this project.

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CHAPTER I

INTRODUCTION

Obesity is a worldwide epidemic, affecting approximately 30% of United States adults (Steinbaum, 2004; Berthoud and Morrison, 2008). Rates of overweight and obesity have been increasing dramatically over the past twenty years and are expected to reach up to 86% by 2030 (Wang et al., 2008). Currently obesity is the second leading cause of death in adults due to its large number of co-morbidities including type 2 diabetes, cardiovascular disease, and cancer (Lehrke and Lazar, 2004; Hotamisligil, 2006). Inflammation is linked to the etiology of some of the obesity-associated diseases, and emerging research suggests that obesity is accompanied by chronic inflammation (Kahn and Flier, 2000; Wellen and Hotamisligil, 2003; Wellen and Hotamisligil, 2005).

A westernized diet, calorically rich and high in saturated fat, is associated with the initiation of inflammatory signaling cascades prior to the onset of obesity (Milanski et al., 2009). Central (hypothalamic) inflammation is of upmost concern due to its potential in disrupting normal signaling in the hypothalamus. Chronic exposure to a high-fat (HF) diet results in increased body weight, increased total adiposity, and energy balance disruptions (Woods et al., 2004). Additionally, these changes are associated with central insulin and leptin resistance. Therefore, the downstream effect of a HF diet-induced inflammatory response may result in the central insulin and leptin resistance that is characteristic of energy balance disruption and obesity.

Pre-menopausal women seem to be protected from the development of inflammatory diseases (Shi and Clegg, 2009). Sex-related differences in disease risks are associated with the major female sex hormone, 17β-estradiol (E2). When presented with a HF diet, female rodents have reduced energy intake and increased energy expenditure compared to males (Shi and Clegg, 2009). E2 has the cellular capabilities to reduce the production of free-radicals, increase free-radical scavenging, and directly inhibit inflammatory signaling (Straub, 2007). By reducing central inflammation, females may in some cases be protected against extreme changes in energy balance that would lead to an obese state.

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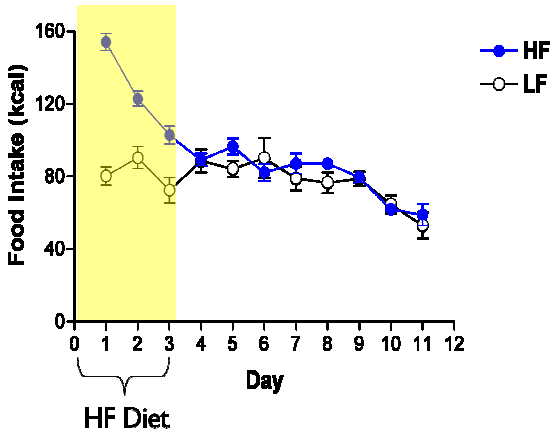
ergy intake during the



While the long-term effects of a HF diet seem clear, the acute changes after a short-term HF diet administration are not. After a standard 24 hour novelty-induced hyperphagia (Figure 1-1), some observations suggest no changes in energy intake compared to chow-fed animals while others have reported a prolonged hyperphagia (Kitraki et al., 2004; Soulis et al., 2005; Banas et al., 2009). Conflicting results also

Figure 1-2. **Hypothalamic Gene Expression in Males-** males on a HF diet have increased pro-inflammatory gene expression

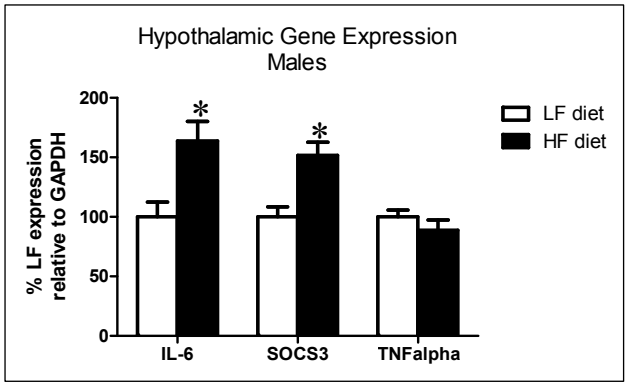
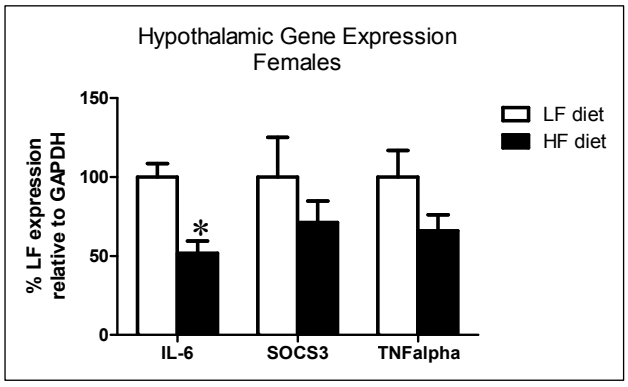


Figure 1-3. **Hypothalamic Gene Expression in Females**- females on a HF diet have decreased pro-inflammatory gene expression



Figures 1-2 and 1-3. Three month-old male and female Long-Evans rats (total 22; n= 4-7/group) were given a LF diet (Harlan-Teklad #7012, Indianapolis, IN; 3.5 kcal/g, 6% fat) or a HF diet (Research Diets, New Brunswick, NJ; 4.54 kcal/g, 40 % fat) for 72 h. Females were phased daily in the middle of the light phase and started on the HF diet on the day of estrous so that 72 h later they would be in proestrus.

exist in body weight and insulin and leptin sensitivity.

It has been demonstrated however that central insulin and leptin resistance may occur after 72 hours on a HF diet (Wang et al., 2001; Morgan et al., 2004). Since inflammatory signaling plays a major role in the inhibition of both the leptin and insulin receptor in the hypothalamus (Zhang et al., 2008), it is possible that the observed resistance after 72 hours on a HF diet may be due to diet-induced inflammation. Preliminary research from our lab suggests that after short-term exposures (72 hours) to a HF diet males have increased inflammation (Figure 1-2), but in females there is a declining trend in pro-inflammatory gene expression (Figure 1-3). Additionally, in male

rats there is an adjustment to the diet that is observed during the first 72 hours of the HF diet (Figure 1-1). The experiments in my thesis project were performed to further understand the trends in inflammatory signaling in short-term exposures to HF diet in male and female rats. Our goals were to examine the sex differences in response to a HF diet by measuring the role of E2 in adiposity, physical activity, and gene expression.

Therefore, the purpose of this research is to determine both the anti-inflammatory capacity and anorectic effects of 17β-estradiol in the early physiological adaptations to a high fat diet prior to the onset of obesity. The central hypothesis is: *17β-estradiol decreases inflammation associated with short-term administrations of a HF diet by blocking signaling through the NFκB pathway, which prevents increased expression of pro-inflammatory cytokines.* This hypothesis was tested in two studies.

Study Aims

Study 1. Determine sex differences between proestrus rats and male rats in HF diet-induced inflammation. 3 month old Long-Evans male and female rats were used in this study. Females were phased daily so that the peak of E2 (proestrus) could be determined. Rats were given a HF diet for 72 hours and euthanized. Females were in proestrus at sacrifice. Based on preliminary data the *working hypothesis* for this aim is that inflammation will increase in male rats given a HF diet compared to female rats in proestrus as a result of activation of the central IKKβ/NFκB pathway.

Study 2. Identify the central anti-inflammatory estrogen pathways that are blocked in HF diet-induced inflammation. 3 month old Long Evans females were used in this study. Ovariectomized (OVX) rats were given E2 or vehicle subcutaneous injections every 4 days to mimic the estrous cycle. The *working hypothesis* for this aim is that inflammation will increase in vehicle-treated OVX rats given a HF diet when compared to E2-treated OVX rats as a result of activation of the central IKKβ/NFκB pathway.

Conclusions

If the specific aims are achieved, this will provide evidence for the anti-inflammatory role of E2 in short-term exposures to a HF diet. Activation of the central IKKβ/NFκB pathway is correlated with leptin and insulin resistance, and diet-induced obesity in rodents. If E2 is capable of blunting inflammatory gene expression caused by the activation of NFκB, this may provide support for reduced susceptibility to develop central leptin and insulin resistance when intact females are fed a HF diet.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Significance

Obesity is a complex disorder reflecting the effect of a network of genes that are influenced by diet, age, sex, and physical activity (Brockmann and Bevova, 2002). Clinical obesity is defined by a body mass index (BMI) of 30 or higher (NHLBI, 1998). Elevated BMI, especially visceral adiposity, increases the risk of hyperinsulinemia, insulin resistance, hypertension, type 2 diabetes, coronary artery disease, and certain cancers (Pi-Sunyer, 2009). These complications have high rates of morbidity and mortality and underscore the importance of identifying people at risk for obesity and its related disorders (Pi-Sunyer, 2009). 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 (Clegg and Woods, 2004; Haslam and James, 2005). Expanding our knowledge of how obesity develops and progresses may lead to preventative and therapeutic treatments to reduce obesity-related mortality and the load placed on our health care system.

Causes of Obesity

It has been theorized that obesity has its origins in different etiologies. The neuroendocrine system regulates food intake, energy expenditure, and body weight.

Drastic changes in neuroendocrine regulation results in dramatic increases or decreases in body weight. Disruptions of this system can occur through injury and viral disease, genetic illnesses, and dietary interactions such as high glucose or fat intake (Hetherington and Ranson, 1940; Anand and Brobeck, 1951; York and Hansen, 1998; Berthoud and Morrison, 2008). While the exact mechanisms for obesity are yet to be elucidated, a complex interaction of genetic traits and the environment are implicated in the prevalence of overweight and obesity.

Genetic Causes

Several theories have emerged to help explain the rising rates of obesity. Thrifty genes have been suggested to provide a genetic cause for obesity (Berthoud and Morrison, 2008). Before the energy-rich times of today, our human ancestors were prone to sporadic times of famine. Thrifty genes promoted increased food intake and energy storage in times of plenty to assist in survival when food became scarce.

Another possible explanation for the increased obesity is genetic drift (Berthoud and Morrison, 2008). A combination of relaxed upper weight limits and reduced threats from predators have resulted in decreased energy expenditure and increased weight. It is possible that the role of genomics in human obesity may be that of predisposition. Genomic disorders that result in obesity only account for a small percentage of today's obese population (Bouchard, 2007; Berthoud and Morrison, 2008). To explain the rapid and alarming increase in obesity, interactions with the environment seem to be the major contributor to its development.

Environmental Causes

The developed world is an obesogenic environment. The plethora of calorically dense, high-fat foods, low energy expenditure, and increased stress has resulted in rapidly expanding waistlines. In particular, the increased intake of saturated fat is obesogenic in both human and rodent models. Dietary fatty acids are able to bind to cell receptors and activate signaling cascades (Milanski et al., 2009). These potential disruptions to normal cellular signaling can affect the pancreas, altering insulin production, as well as, appetite control sensors in the brain. Diets high in saturated fat can increase low density lipoprotein (LDL) levels and increase blood pressure (Riccardi et al., 2004).

Commonly Used Rodent Models of Obesity

Genetic Models

Many genes are involved in food intake and energy metabolism. These include genes for adipocyte regulatory proteins, circulating factors, mitochondrial proteins, cell surface receptors, and neuropeptides (Campfield et al., 1998). Several have been identified using knockout or transgenic mouse models where genetic manipulation has resulted in body weight changes accompanied by altered expression of downstream genes (Good, 2000). A commonly used example for obesity research is the ob/ob mouse (Zhang et al.). Ob/ob mice are unable to create leptin which is the cause for the obesity in this model. Ob/ob mice are hyperphagic, hyperinsulinemic, hypothermic, insulin resistant and have increased glucocorticoid production (York and Hansen, 1998) which is measurable in early life stages. Leptin, an adipocyte derived hormone, exerts appetite suppressing effects when it interacts with the long form of the leptin receptor (Ob-Rb) in the

hypothalamus. An inability to respond or make leptin results in hyperphagia and obesity. Resistance to the effects of leptin is a commonality in rodent models of obesity and has been suggested to occur in humans (Berthoud and Morrison, 2008). However, genetic errors in leptin production or signaling are not commonly seen in humans (Speakman et al., 2007). While the discovery of the ob/ob mouse was an important landmark in obesity research by elucidating the importance of leptin for controlling adiposity, it does not mimic the environmental factors that contribute to the obesity commonly seen in humans.

Diet-Induced Obesity

Dietary manipulations like force-feeding, polycose solutions, high fat, high sucrose diets and even laboratory chow have been used to induce obesity (York and Hansen, 1998). The variable success of these manipulations across rodent strains suggests that environmental changes are interacting along different genotypes with differing results. The interaction of genetic susceptibility and environmental opportunity are important to produce dietary obesity. Diet-induced obesity (DIO) models are used to mimic what is commonly seen in human obesity progression (Reuter, 2007; Speakman et al., 2007; Gajda, 2008). Dietary fat composition is manipulated to spur an increase in weight gain and adiposity. These diets contain fat levels ranging from 32-60% (Reuter, 2007; Gajda, 2008) compared to 5-20% in a standard laboratory chow (Reuter, 2007). Additionally, these diets contain larger amounts of saturated fat which is obesogenic, highly consumed in the developed world, and associated with increased disease. Commonly used strains for DIO include the Long-Evans rat, Sprague-Dawley rat, and C57/BL6J mouse (Reuter, 2007; Speakman et al., 2007). Overall, genetically

manipulated models allow for the investigation of the specific roles of the affected neuropeptides and signaling cascades, whereas DIO models allow us to study of a progression to an obese state that is most characteristic of human obesity (Woods, 2005).

Energy Balance

As discussed previously, the environment plays an important role in the development of obesity. The calories that are consumed throughout the day should be close to the amount that is expended to stay in balance. If an increase in calories is consumed, it must be met with increased energy expenditure or decreased food intake in the future. If the body does not adapt to the influx of energy, there will be increased fat storage and subsequent weight gain. Overall, both rodents and humans have an innate ability to maintain adiposity over a long period of time. Information on energy needs is transmitted through hormonal and vagal nerve input from adipose tissue and digestive organs (Berthoud and Morrison, 2008). These signals are integrated in brain areas like the hypothalamus resulting in changes in food intake and energy expenditure.

Hypothalamic Control of Energy Balance

Both anorexigenic and orexigenic nerve fibers run through the arcuate nucleus (ARC) of the hypothalamus (Woods, 2005). Hormonal stimuli from the body can interact with these nerves resulting in expression and release of neuropeptides. The most influential of the anorexic hormonal cues include leptin and insulin. 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. Immediate energy needs can drive food intake through the release of hormones from the gastrointestinal (GI) tract (Woods, 2005; Milanski et al., 2009). Ghrelin, an orexigenic hormone released from the stomach, acts on the hypothalamus. The hypothalamus receives information about energy status from other brain regions like the brain stem and it receives direct inputs from plasma nutrients including glucose and fat.

In the ARC the appetite-suppressing hormones such as leptin and insulin, interact with pre-opiomelanocortin (POMC) neurons. POMC can be cleaved to produce the anorexigenic neuropeptide alpha-melanocyte-stimulating hormone (α-MSH) (Woods, 2005; Berthoud and Morrison, 2008). In opposition, ghrelin can stimulate neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons to increase food intake. ARC neurons project to other hypothalamic regions para-ventricular nucleus (PVN) and the lateral hypothalamus (LHA) to regulate food intake. Additionally, cross talk between hypothalamic neurons can result in inhibition or more dramatic responses to orexogenic signals (Berthoud and Morrison, 2008).

Human obesity is characterized by increased consumption of high-fat, energy dense foods. A high consumption of fat, especially saturated fat, disrupts hypothalamic signaling. Long-term exposure to a high-fat (HF) diet in rodents reduces hypothalamic Ob-Rb expression, resulting in leptin resistance and obesity (Heshka and Jones, 2001). These changes in Ob-Rb expression are associated with reductions in POMC gene expression, which may be part of the mechanism for the changes in food intake and energy expenditure seen in DIO animals. In addition to blunting Ob-Rb expression, HF

diets have also been suggested to directly affect cell membrane motility (Heshka and Jones, 2001). It has recently been demonstrated that a diet high in saturated fats can result in increased saturated fat incorporation into the cell membrane. This can cause significant issues for membrane receptors because the cell membrane is mostly made up of polyunsaturated phospholipids. An increased incorporation of saturated fatty acids in the membrane results in reduced fluidity, which may alter Ob-Rb functioning. While the connections between HF diets and the development of obesity is still being ascertained, it has been demonstrated that leptin resistance is a causative factor. Leptin resistance leads to weight gain and obesity.

Long-term exposure to a HF diet, up to 70 days in some studies, leads to obesity in rodents that is accompanied with a significant increase in food intake, body weight, and body fat (Woods et al., 2004; Gajda, 2008). While a prolonged intake of HF diet causes hyperphagia and obesity, the effects of shorter exposures to HF diets are not as clear. Some studies have suggested a prolonged hyperphagia beyond the initial 24 hours (Banas et al., 2009) where others have found reduced food intake compared to chow fed animals after 7 days (Kitraki et al., 2004; Soulis et al., 2005). The later studies attributed the observed reductions in food intake and body weight to compensatory mechanisms including increases in serum leptin levels and retained central leptin sensitivity. Conversely, other studies (Wang et al., 2001) have observed both leptin and insulin resistance after 72 hours on a HF diet. Wang et al. reported significant increases in food intake and body weight in HF diet-fed animals compared to controls after 3 and 7 days on a HF diet. Slightly longer studies, up to 4 weeks, have reported decreased serum leptin

levels after HF diet but with no observed difference in body weight or energy intake (Ainslie et al., 2000). Therefore it is important to clarify the large differences observed in energy balance and weight after a short-term challenge to a HF diet.

The Role of Spontaneous Physical Activity in Energy Balance

The other half of the energy balance equation is energy expenditure, which includes basal metabolic rate, the thermic effect of food, and physical activity. The amount of daily physical activity performed is further classified into physical activity for sport and spontaneous physical activity (SPA) (Tou and Wade, 2002). SPA, which compromises non-exercise-associated thermogenesis, includes activities of daily living such as walking, grooming, and fidgeting but does not include activities like eating or sleeping (Tou and Wade, 2002; Castaneda et al., 2005). Studies have suggested that up to 60% of total daily calories expended through physical activity are from SPA. In humans, reduced levels of SPA is a strong predictor of subsequent weight gain and obesity (Tou and Wade, 2002; Kotz et al., 2008). Research suggests that the same hormones and neuropeptides that regulate food intake in the hypothalamus can also regulate the levels of SPA (Castaneda et al., 2005). Direct injection of ghrelin and AgRP into the third ventricle of the hypothalamus in rodents results in a significant reduction of SPA levels. Additionally, leptin administration in ob/ob mice resulted in increased SPA prior to the onset of obesity. These findings, particularly having to do with obesity progression, suggest that SPA can play a major role in the prevention of obesity.

The dysregulation of food intake found when rats are presented with a HF diet is also present in regards to SPA. Short increases in food intake result in a significant

increase in SPA (Castaneda et al., 2005), potentially as a compensatory mechanism, whereas a longer exposure to HF diet reduces levels of SPA. Novak et al. found that rats fed a HF diet for 1 month had a significant reduction in total activity levels compared to rats that were resistant to HF diet-induced obesity. The authors suggested that the reduction in SPA was a precursor to weight gain and obesity (Novak et al., 2006). In addition to reductions in overall SPA, it has also been suggested that HF diet can disrupt normal circadian rhythms of activity. Kohsaka et al. found that mice given a HF diet for 6 weeks reduced dark cycle activity and only slightly increased in light cycle activity. Taken together, these findings suggest that total activity is reduced when rodents are fed a HF diet (Kohsaka et al., 2007). In addition to dietary manipulation of both SPA and food intake, it has also been suggested that other variables can influence these behaviors such as certain diseases, medications, age, and sex (Tou and Wade, 2002).

Commonly Used Ways to Measure Spontaneous Physical Activity

A large amount of previous literature, both before and after the introduction of computer software, relied on the use of the home cage running wheel to measure SPA. Unfortunately, current research suggests that animals that have access to running wheels will significantly increase their SPA regardless of dietary manipulation (Novak et al., 2006; Basterfield et al., 2009). These findings suggest that just the exposure to a running wheel will result in increased activity and therefore should not be used to determine SPA levels in rodents. Advances in software engineering have permitted more specific measurement of rodents with little to no changes in novelty, therefore reducing the amount of anxiety in rodents. These programs use laser beams projected vertically and

horizontally, creating a 3 dimensional plane, so that movement is measured by beam breaks. Studies incorporating this equipment provide information on behaviors including total distance traveled and rearing. Unfortunately such programs are limited to the amount of behaviors it can determine such as eating and drinking.

As the knowledge of computers, software, and animal behaviors advances, future equipment can increase the accuracy and specificity of behavior reporting and increase the amount of data collected. One such program that significantly increases the quality and quantity of behaviors measured is the HomeCage Scan by Clever Systems, Inc. Additionally, some investigators have also built their own equipment to measure SPA (Blanchard et al., 1995; Atchley and Eckel, 2005). While this may provide the ability to develop equipment that is tailored to the needs of a particular protocol, its overall accuracy is difficult to determine and the results from such studies may be hard to compare to others that use different equipment or software.

Sex Differences

Rats and mice have also shown substantial individual variability in their susceptibility to diet-induced obesity (West, 1996; Levin et al., 1997; West and York, 1998). While a variety of mechanisms may contribute to this observed variability, differences in strain and sex, as well as other genetic or developmentally programmed differences in energy balance could be involved (Bouret and Simerly, 2006; Speakman, 2007; Gluckman et al., 2008). The effects of sex on energy balance and adiposity is significant. Females tend to have a decreased risk of developing inflammatory-related

diseases including CVD and cancer, and potentially obesity (Shi and Clegg, 2009). This decreased risk is likely due to the ovarian hormone, 17β-estradiol (E2).

The Effects of Estrogen on Body Composition

The major female hormone, E2, strongly influences were animals carry fat. Estrogens can produce non-genomic changes through interaction with a G-coupled protein receptor (GPR30) on cellular membranes (Revankar et al., 2005), whereas genomic changes are caused by estrogen receptors. The estrogen receptor is a steroid receptor that serves as an active transcription factor when bound to E2. Two isoforms have been identified and include estrogen receptors alpha (α) and beta (β) (Toft and Gorski, 1966; Kuiper et al., 1996). While both actively induce changes in gene expression, most of the sex differences observed in metabolism are attributed to the interaction between E2 and estrogen receptor α (Imamov et al., 2005).

Both pre-menopausal women and rodents tend to store fat subcutaneously, while males of both species have larger visceral fat stores. Visceral fat is more metabolically active and is associated with increased risk for the metabolic syndrome (Shi and Clegg, 2009). Due to the increased metabolic activity of these adipocytes, a higher amount of visceral fat mass is associated with increased risk of inflammatory related diseases such as cardiovascular disease (CVD) and certain cancers, like colon and breast cancer. This may help explain the increased occurrence of CVD and the metabolic syndrome in men compared to pre-menopausal women. The difference in fat deposition between the sexes may be due to the action of E2 on the level of the adipocyte (Shi and Clegg, 2009). E2 increases lipolytic capacity of visceral adipocytes by increasing their sensitivity to leptin.

In addition to working directly on the adipocyte, E2 can regulate energy balance through its interactions with anorexic and orexic neuropeptides in the hypothalamus like NPY, AgRP and POMC (Brown and Clegg, 2009; Shi and Clegg, 2009).

Estrogen Effects Energy Balance through CNS Regulation

Many investigators report that female rodents have reduced food intake and higher amounts of SPA compared to male rodents (Lightfoot, 2008; Brown and Clegg, 2009; Shi and Clegg, 2009). Estrogen receptor alpha α is highly concentrated in the hypothalamus. The E2-estrogen receptor α complex induces genomic and non-genomic changes in neurons. Shortly after administration of E2 in the hypothalamus, an increase in the release of POMC occurs (Kelly and Ronnekleiv, 2009). E2's genomic actions include an increase in Ob-Rb expression (Shi and Clegg, 2009). As stated earlier, leptin is secreted in proportion to subcutaneous fat mass. Females, having larger amounts of subcutaneous fat, secrete more leptin than males. Since E2 increases Ob-Rb, this mechanism may explain why females are more sensitive to the anorexic effects of leptin. Additionally, intact female rodents have differing amounts of circulating E2 depending on the day of the estrous cycle. The levels of Ob-Rb expressed in the ARC fluctuate in response to the estrous cycle (Shi and Clegg, 2009). Rodents in proestrus, when blood levels of E2 are at their peak, have higher amounts of Ob-Rb compared to rodents in other phases. This may be the mechanism for the reduction of food intake and body weight in proestrus rats (Shi and Clegg, 2009) and the increases in SPA (Tou and Wade, 2002), since there is increased leptin sensitivity during this time.

The effects of a HF diet in female rodents are similar to what is seen in males, though the changes are suggested to be significantly lower than in males (Hong et al., 2009). Hong and colleagues reported that female mice gain less after 20 weeks on a HF diet than males. Additionally, the body weight change in HF diet-fed females mimicked that of chow-fed males (Hong et al., 2009). This suggests a potential protection from obesity and weight gain. Although this study did not investigate behaviors, the difference in weight and body fat that was observed may be due to sex differences in food intake and SPA. Both males and female rats display increased energy intake when given a HF diet compared to chow-fed controls (Priego et al., 2009). Between the HF diet groups, females seem to be more susceptible to a diet-induced hyperphagia but overall display decreased energy intake compared to males (Priego et al., 2009). The reduced daily energy intake in females resulted in a significant decrease in body weight gain compared to HF diet-fed males. These results are to be expected due to the central leptin and insulin resistance that is associated with DIO. Additionally they suggest that females may be protected from DIO even though they are slightly more hyperphagic than males. In regards to SPA, Basterfield et al. found that female C57BL/6J mice given a HF diet for 8 weeks increased the total amount they slept in 24 hours and decreased SPA (Basterfield et al., 2009). Interestingly, the findings of this study mimics what was seen in HF diet male mice by Kohsaka et al. discussed previously. Ultimately it appears that HF diet results in reductions in SPA in both sexes compared to their LF diet-fed controls. While females display an increased diet-induced hyperphagia compared to males, they still have a reduced total energy intake and do not become obese in many DIO paradigms (Kohsaka

et al., 2007). Unfortunately, findings are not as clear on SPA since no published research currently exists comparing males and females on a HF diet. After reviewing the literature on food intake and body weight, it is possible that intact females may display some protection to the SPA reductions and contributes further to their reduced susceptibility to become obese on a HF diet.

The Effects of Ovariectomy on Energy Balance

During rat proestrus, both progesterone and E2 are at their peak. To determine the sole effects of E2, ovariectomy (OVX) is often used. The procedure of OVX, which entails surgical removal of both ovaries, results in an overwhelming reduction of E2 synthesis and secretion. Immediately after OVX, rodents experience a dramatic increase in body weight and visceral fat deposition (Shi and Clegg, 2009). These changes are accompanied with reductions of SPA, particularly during the dark phase (Rogers et al., 2009). E2 replacement after OVX results in a reduction in total adiposity as well as a return to normal levels of daily energy intake and energy expenditure (Leshner and Collier, 1973; Wade and Gray, 1979; Wade et al., 1985).

When OVX rodents are given a HF diet, they gain weight similarly to age-matched males (Hong et al., 2009). They have disrupted meal patterns, larger meal size, and increased food intake compared to intact female rodents (Hong et al., 2009). While intact females increased food intake and body weight, it is significantly lower than males or OVX rats. E2 replacement reverses the increases in energy intake and body weight caused by a HF diet. Unfortunately, very little research exists on the effects of HF diet

and OVX on SPA. Therefore it is difficult to determine how much E2 replacement may blunt the changes of SPA caused by a HF diet.

Methods of Estrogen Replacement

There are several ways to replace E2 in OVX rats. Investigators may implant a pellet subcutaneously that will continually release E2. This method allows for a constant, controlled level of circulating E2 throughout the body and it is often used in chronic E2 treatment paradigms. Additionally, selective estrogen receptor modulators are used to activate or block estrogen receptors when short-term disruptions in E2 activity are needed. Intact females do not maintain constant levels of estrogen. During the estrous cycle E2 is only at its peak during proestrus, while it is low on the other three days of the cycle. Therefore, the use of pellets for estrogen replacement is not necessarily the most physiologically adaptive.

In order to mimic the cycle of intact female rats, cyclic estrogen replacement design is needed. Previous studies attempting to use a cyclic replacement design did not fully mimic what is observed (reduced body weight and food intake) in intact rats (Tarttelin and Gorski, 1973; Geary and Asarian, 1999). Tarttelin and Groski for example delivered 3 μg of E2 every 5 days; this design may have delivered too much E2, which was spread too far apart. Asarian and Geary were the first to use a cyclic E2 replacement paradigm that results in a plasma E2 level that equals that in intact females. E2 injected subcutaneously at 2 μg every 4 days produced the classic E2 spike at similar plasma levels of intact rats. Additionally, this treatment paradigm also produced the same

behavioral changes (reduced food intake and body weight gain) that occurs in intact females (Asarian and Geary, 2002).

Inflammation

Inflammation plays a major role in the development of obesity (Zhang et al., 2008; Kleinridders et al., 2009). While the inflammatory process is a life-saving mechanism to reduce invading microbes and aid in healing, chronic inflammation is deadly to cells (Nathan, 2008). Chronic inflammation is present in obese people as determined by the higher blood cytokine levels (Nathan, 2008).

Potential Mechanisms for High Fat Diet-Induced Inflammation

Chronic inflammation activates IKKβ/NFκB signaling in the hypothalamus resulting in resistance to insulin and leptin. In contrast, suppression of 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 suppressor of cytokine signaling 3 (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). While it is increasingly recognized that inflammation is an important factor in the incidence of type 2 diabetes and obesity (Lehrke and Lazar, 2004; Hotamisligil, 2006), the connection between inflammation and dysfunctional signaling in the hypothalamus is not fully understood.

Endoplasmic Reticulum Stress Coordinates High-Fat Induced Inflammation

Free fatty acids (FFA) bind to membrane receptors and increase inflammatory signaling cascades (Milanski et al., 2009). Additionally, an increased level of dietary fat increases the production of reactive oxygen species (ROS), which can stimulate inflammatory signaling (Nathan, 2008). FFAs and cellular ROS also result in the formation of endoplasmic reticulum (ER) stress, which is elevated in obese and insulin resistant rodents (Marciniak and Ron, 2006). FFAs cause hypothalamic inflammation and increase cytokine expression by interacting with the toll-like receptor 4 (TLR-4) (Milanski et al., 2009). Investigators also determined that the induction of ER stress after saturated fat intake was dependent on its interaction with TLR-4 (Milanski et al., 2009). Through this receptor, FFAs can cause an accumulation of misfolded proteins that result in a signaling response called the unfolded protein response (UPR). Increased levels of unfolded proteins directly activate the inositol-requiring 1 (IRE-1) and tumor necrosis factor receptor-associated factor 2 (TRAF-2) receptor complex which leads to nuclear factor kappa B (NFκB) and c-jun N-terminal kinase (JNK) activation (Yang and Hotamisligil, 2008; Zhang and Kaufman, 2008; Zhang et al., 2008). Activation of ER stress signaling and its subsequent NFκB activation have been demonstrated in HF diet-induced hypothalamic inflammation (Zhang and Kaufman, 2008; Zhang et al., 2008). While both NFκB and JNK act as transcription factors, activating gene expression of inflammatory cytokines such as tumor necrosis factor α (TNFα) and interlukin 6 (IL-6), they also influence hypothalamic sensitivity to appetite regulating neuropeptides. JNK is able to blunt insulin signaling by inactivating insulin receptor substrate 1 (IRS-1) through

serine phosphorylation (De Souza et al., 2005; Yang and Hotamisligil, 2008). NFκB has been shown to induce SOCS3 gene expression (Zhang et al., 2008), which can cause leptin resistance by blocking Ob-Rb (Yang and Hotamisligil, 2008). Taken together these findings suggest a complex interaction between body systems and cellular organelles resulting in chronic inflammation and body weight gain through central leptin and insulin resistance.

Hypothalamic Inflammation Results in Disruption of Energy Balance

There are changes in energy balance that precede the onset of obesity when animals are fed 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 body weight in rats fed a HF 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). Little research examining hypothalamic inflammation and SPA levels has been published. While LPS-induced inflammation reduces SPA levels, it also causes hypophagia and weight loss (Franklin et al., 2003). Therefore this model is not representative of HF diet-induced inflammation, but it does further support that the hypothalamus regulates SPA and that this may be disrupted by inflammation. Further research is needed to determine if inhibition of inflammatory signaling in HF diet rodents results in restoration of SPA levels.

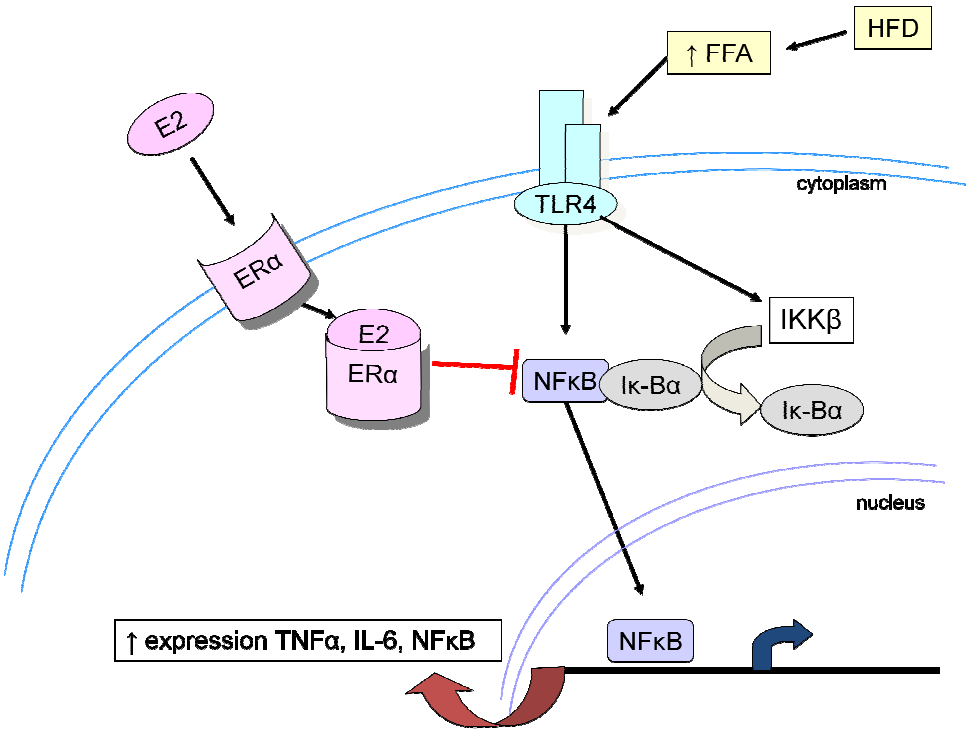
Estrogen as an Anti-inflammatory may Blunt the Effects of a HF Diet

Pro-inflammatory cytokines in the brain perform many functions and are synthesized by microglia, astrocytes, and neurons (Hanisch, 2002). In several brain injury paradigms, E2 suppresses pro-inflammatory cytokines and increases the production of anti-inflammatory cytokines (Matejuk et al., 2001; Salem, 2004). In stroke models, increased levels of anti-inflammatory cytokines have also been linked to a reduction in stroke severity (Acalovschi et al., 2003; Mergenthaler et al., 2004). TNFα (Hallenbeck, 2002) and IL-6 (Acalovschi et al., 2003) are mediators of neuronal survival that play important roles in the inflammatory response.

E2 may have a role in reducing the inflammatory response in adipose, cardiovascular, and neural systems (Turgeon et al., 2006), in addition to being neuroprotective (Vegeto et al., 2001; Vegeto et al., 2003; Vegeto et al., 2006). Estrogen receptor α (and in some cases estrogen receptor β) 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). The anti-inflammatory properties of E2 can 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, IL-6, and IL-8 (Straub, 2007; Hamilton et al., 2008). In addition to changes in serum cytokines during the estrous cycle, OVX is associated with an increased cytokine expression that is reversible upon E2 treatment (Evans et al., 2001; Hamilton et al., 2008). 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 NADPH oxidase thereby increasing the cellular capacity of free-radical scavenging and reducing formation of ROS respectively (Straub, 2007). Additionally, there is much research on the ability of E2 and estrogen receptor α to regulate NFκB activity (Stice and Knowlton, 2008) (Figure 2-1). Genomically, E2 is able to increase the expression of IkBα, the inhibitory subunit of NFκB. E2 can also reduce IkBα phosphorylation, keeping it bound to NFκB and preventing the activation of NFκB. 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 (Evans et al., 2001).

Figure 2-1. **E2 Blunts NFκB Signaling**- E2 interacts with NFκB preventing its activity and downstream gene expression.



l-like receptor
ignaling 3;

Estrogen May Control Inflammatory Signaling by Managing ER Stress

Lastly, E2 may also regulate inflammatory signaling by managing the ER stress response. When misfolded proteins accumulate in the ER lumen, IRE-1 activates XBP1 protein through unconventional splicing. Once activated, XBP1 serves as a transcription factor for chaperone proteins, which reduces cellular stress (Lee et al., 2003). XBP1 deficiency in HF diet-fed mice results in rapid weight gain (Ozcan et al., 2004; Ozcan et

al., 2009) and systemic insulin resistance (Ozcan et al., 2004). This observation is hypothesized to be the result of uncontrolled inflammatory signaling due to HF diet-induced ER stress.

There is an estrogen response element (ERE) on the promoter region of the XBP1 gene (Carroll et al., 2005). Additionally, unbound heat shock proteins, like those that are released when E2 binds to its intracellular receptor, are capable of reducing ER stress by binding to unfolded proteins and assisting in their degradation (Stice and Knowlton, 2008). A reduction of unfolded proteins reduces of IRE-1, NFκB, and JNK activation. These data taken together may provide a mechanism for a reduction in inflammation and ER stress observed with E2.

Conclusions

An inflammatory process that results from increased FFAs may cause DIO. Research suggests that the inflammatory signaling cascade that results from overnutrition can lead to leptin and insulin resistance, both potent anorexogenic hormones that are important for energy balance. Increased food intake and body weight is associated with hypothalamic inflammatory signaling, and it is possible that E2 has the ability to reverse this by decreasing inflammation. If inflammation is the cause of energy balance dysregulation, this could provide targets for obesity treatment.

Pre-menopausal women have some protection from inflammation-related disease until they reach menopause, when their risks for developing obesity and the metabolic syndrome equals that of men. Female rodents, as well, display this same protection in disease models of obesity and diabetes. It is possible that this protection may be due to

the anti-inflammatory effects and cellular stress reducing effects of E2. Additional research is needed to fully elucidate the mechanisms of this observed protection against the development of diet-induced inflammation and obesity.

CHAPTER III

PROESTRUS FEMALES HAVE REDUCED INFLAMMATORY STRESS AFTER 72 HOURS ON A HIGH-FAT DIET

Abstract

There is evidence that obesity is characterized by chronic activation of inflammatory pathways. The protective effects of ovarian hormones may be a result of the anti-inflammatory effects of estradiol and progesterone in the hypothalamus and liver. In the present study we sought to determine if this effect is present when a high-fat (HF) diet is first introduced. Age-matched male and female Long-Evans rats (three-months old) were given a HF or a low-fat (LF) diet for 72 h (n= 90). Females were phased daily and started on the HF diet on the day of estrous so that they would be in proestrus at sacrifice. The liver and hypothalamus were extracted and processed using quantitative PCR of IL-6, SOCS3, TNFα, and XBP1. Males on the HF diet had increased SOCS3 expression in the hypothalamus compared to their LF controls. However, in females there was a reduction in inflammatory gene expression between diet groups. Endoplasmic reticulum (ER) stress, measured by higher expression of XBP1, was reduced in females on the HF diet compared the LF controls. However a sex difference in ER stress did exist. Males fed HF diet had higher XBP1 expression than their female counterparts in the liver. These data provide support for the protective role of ovarian hormones in inflammatory diseases.

Introduction

Obesity is an epidemic effecting approximately 30% of United States adults (Steinbaum, 2004; Morrison and Berthoud, 2007). Rates of overweight and obesity have increased dramatically over the past twenty years and are expected to reach up to 86% by 2030 (Wang et al., 2008). Currently, obesity is the second leading cause of preventable death in adults due to its co-morbidities including type 2 diabetes, cardiovascular disease, and cancer (Lehrke and Lazar, 2004; Hotamisligil, 2006). Consumption of food that is high in fat and calorically dense is implicated as one of the most important environmental factors leading to obesity (Woods et al., 2004). Ingestion of a high-fat (HF) diet leads to hypothalamic leptin and insulin resistance (Clegg et al., 2003; Mori et al., 2004). However, the mechanisms responsible for activating inflammatory signaling pathways in obesity are poorly understood.

Inflammation has been linked to the etiology of some of the obesity-associated diseases, but emerging research suggests that the development of obesity requires a systemic inflammatory state (Kahn and Flier, 2000; Wellen and Hotamisligil, 2003; Wellen and Hotamisligil, 2005). A westernized diet, calorically dense and high in saturated fat, is associated with the initiation of inflammatory signaling cascades. Saturated fat has shown to stimulate inflammatory signaling and cellular stress in rodent models of diet-induced obesity (DIO) (Nathan, 2008; Milanski et al., 2009). Both the insulin and leptin receptors are sensitive to intracellular stress and can be inactivated by inflammatory signaling (Zhang et al., 2008). CNS inflammation disrupts normal

signaling in brain satiety centers, particularly in the hypothalamus (De Souza et al., 2005; Yang and Hotamisligil, 2008; Zhang et al., 2008).

Chronic inflammation caused by high fat diets activates IKKβ/NFκB signaling in the hypothalamus that results in resistance to insulin and leptin. In contrast, suppression of IKKβ in the medial basal hypothalamus, or in hypothalamic AgRP neurons, reverses obesity (Zhang et al., 2008; Kleinridders et al., 2009). The molecular mechanisms involved in these processes include the interaction of SOCS3 with NFκB, with SOCS3 being an important inhibitor of insulin and leptin signaling. Signaling by the IKKβ/NFκB pathway in the hypothalamus represents an important factor in obesity, and correspondingly it has been proposed that suppression of hypothalamic NFκB signaling may inhibit obesity and related diseases (Zhang et al., 2008; Kleinridders et al., 2009).

The risk of inflammatory-related diseases is reduced in premenopausal women (Shi and Clegg, 2009). Sex differences in disease risks are suggested to be caused by the major female sex hormone, 17β-estradiol (E2). When given a HF diet, female rodents have reduced energy intake compared to males (Priego et al., 2009). These differences in energy balance result in reduced weight gain in female models of DIO. When endogenous E2 is removed from female rodents, primarily through ovariectomy, the protection from HF diet-induced weight and fat gain is attenuated (Shi and Clegg, 2009).

E2 has protective actions in many different tissues. In addition to reducing the production and availability of free-radicals (Straub, 2007), E2 has anti-inflammatory properties (Straub, 2007; Hamilton et al., 2008). E2 disrupts inflammatory signaling

primarily through interacting with the NFκB complex during cellular stress (Stice and Knowlton, 2008). By this mechanism, it is suggested that females may be protected against HF diet-induced cellular stress. Additionally, this may explain the reduced incidence of metabolic disease and cardiovascular disease in female rodents compared to males (Brown and Clegg, 2009; Shi and Clegg, 2009).

Recently, the endoplasmic reticulum (ER) has been targeted as a major regulator of HF-diet induced inflammatory stress. Through the TLR4, saturated fat has shown to activate the unfolded protein arm (UPR) of the ER stress response that can lead to the activation of both NFκB and JNK (Milanski et al., 2009). Additionally, ER stress activation has been linked to suppression of both leptin and insulin signaling *in vivo* (Ozcan et al., 2004; Zhang et al., 2008; Ozcan et al., 2009). A study investigating the x-box binding protein 1 (XBP1) gene located an estrogen response element on its promoter region (Carroll et al., 2005). XBP1 becomes activated by unconventional splicing during the UPR. Once activated it is able to initiate the transcription of chaperone proteins that assist in cell survival. XBP1 must become activated when the cell is undergoing stress, otherwise it will lead to uncontrolled inflammatory cascades and apoptosis. Knockdown of XBP1 results in weight gain and diabetes in rodents that are fed a HF diet (Ozcan et al., 2004). Therefore, another way E2 may be preventing NFκB activity is by controlling HF diet-induced ER stress.

While the long-term effects of a HF diet seem clear, the acute changes after a short-term HF diet administration are not. In some studies, central leptin and insulin

resistance is observed after 72 hours on a HF diet (Wang et al., 2001; Morgan et al., 2004). Inflammatory signaling can result in central resistance to anorexogenic hormones, research is needed to investigate the role of inflammation after in short exposures to HF diet. To investigate this, we selected three genes (SOCS3, TNFα, IL-6) that are responsive to NFκB activation to determine both central and systemic inflammation. Additionally, research suggests that ER stress plays an important role in inflammatory signaling that results in leptin resistance (Zhang et al., 2008; Ozcan et al., 2009). Therefore we also chose to investigate changes in XBP1 expression to serve as an ER stress marker. Lastly, we investigated behavioral differences to determine the dysregulation that occurs when a HF diet is first introduced and to the level to which both male and female rats are able to adjust to saturated FFAs at 24 and 72 hours.

Materials and Methods

Animals

3 month old male (n=44) and female (n=46) Long-Evans rats were purchased from Harlan Labs. 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, females and males were maintained on a standard laboratory chow (17% fat and 3.1 kcal/g, Harlan Teklad #7012; Indianapolis, IN). Rats had free access to food and water *ad libitum* throughout the experiment. Rooms were temperature (22 $\pm$ 2 °C) and humidity controlled and kept on a 12:12 light/dark cycle (lights on at 4 am). At the start of the experiment, a subset of each sex was switched to a high-fat diet (40% fat and 4.54 kcal/g, Research Diets #D03082706; New Brunswick,NJ) (Table 3-1). This HF diet uses butter

as the fat source. It was selected to match the major source of fat in the US diet. The University of North Carolina at Greensboro Institutional Animal Care and Use Committee approved all protocols for this experiment.

Table 3-1. **Diet Information**

Dietary Components	LF Diet	HF Diet
	% total kcal	% total kcal
Carbohydrate	58%	45%
Protein	25%	15%
Fat	17%	40%
Total kcal/g	3.1	4.54

Determination of Estrous Cycling

Female rats were phased daily by vaginal lavage as previously described by Becker (Becker et al., 2005). Daily phasing occurred in the middle of the light cycle, which is the optimal time to determine proestrus through physiological examination. 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.

Behavioral Testing

Measurements of home cage behaviors were performed through real-time video surveillance and HomeCage Scan software (Clever Systems, Inc; Reston, VA). The room was set up with a dark blue background and a red light under each cage for monitoring movement of each animal during the night cycle. Animals were given a 1 day acclimation period to the behavioral room with the video cameras running prior to the start of the 72

hour study. Cages were changed daily in order to reduce the amount of potential interference around the rat and 24 hour behavioral data was downloaded to external hard drives daily. The video segment containing the light to dark transition was saved and transferred onto DVDs. Variables recorded included ambulatory behaviors, exploratory behaviors, rearing behaviors, eating, drinking, and sleeping in both seconds and number of bouts. Food intake and body weight change was measured by subtracting the final food weight and body weights at the time of sacrifice from the starting food weight and body weight at the beginning of the experiment.

Plasma Analysis

After 72 hours on their experimental diet the rats were sacrificed by decapitation. Trunk blood was collected in heparinized tubes and centrifuged. Aliqouts of plasma were collected and stored at –80 °C until analyzed. Plasma leptin was measured using a rat leptin radioimmunoassay (RIA) kit (Linco Research, St. Charles, Missouri). This assay is able to detect leptin in 100 µl samples of plasma. Plasma insulin will be measured by ELISA using a Labsystems Multiscan Plus plate reader (Fisher Scientific; Pittsburgh, PA). Serum concentrations of estradiol will be measured by specific radioimmunoassay (Quest Diagnostics, Inc.-Nichols Institute Diagnostics, San Juan Capistrano, CA).

Inflammatory Gene Expression

At sacrifice, the medial basal hypothalamus and a section of liver were 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: city, state) according to the manufacturer instructions. RNA concentration and purity was assessed by Nanodrop

spectrophotometer (Thermo Scientific, ND-1000; Wilmington, DE). 2ng 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 Real Time-PCR for TNF-α, SOCS-3, IL-6, and XBP-1 using primers from Applied Biosystems (Table 3-2).

Table 3-2: **Applied Biosystems Primers for qPCR**

PRIMERS	Product Number
TNFα	Rn1525860_g1
SOCS3	Rn00585674_s1
IL-6	Rn99999011_m1
XBP1	Rn01752569_m1
GAPDH	Rn01462662_g1

Body Composition

After sacrifice, the skin and subcutaneous fat (pelt) was dissected from the muscle wall and visceral fat (carcass) and stored at -20 °C until analyzed by Dual Energy X-Ray Absorptiometry scan. Both the pelt and body were scanned in duplicate to determine % body fat and % lean body mass.

Statistical Analysis

Statistical analyses were performed using SPSS (version 17.0). To analyze specific planned comparisons involving diet conditions and sex groups, independent t-tests were performed. Significance was set at $p<0.05$; data results are presented as means with corresponding SEMs.

Results

Table 3-3. **Group Food Intake, Body Weight and Composition**

	LF diet Males	**HF diet Males**	**LF diet Females**	**HF diet Females**
72 h FI (kcal)	304.72 ± 9.63^{a}	408.54 ± 12.51^{be}	207.92 ± 3.45^{c}	372.52 ± 21.41^{de}
72 h BWΔ (g)	4.77 ± 4.97^{a}	15.88 ± 9.52^{ab}	1.60 ± 0.98^{a}	16.39 ± 1.25^{b}
Carcass BF%	19.13 ± 0.85^{a}	23.40 ± 1.05^{b}	25.84 ± 1.43^{bc}	28.32 ± 0.82^{c}
Carcass Fat (g)	54.80 ± 3.71^{a}	74.87 ± 4.91^{b}	55.86 ± 3.01^{a}	63.86 ± 2.62^{a}
LBM (g)	230.00 ± 9.17^{a}	242.93 ± 6.82^{a}	161.43 ± 4.65^{b}	160.71 ± 2.83^{b}
Carcass Fluid (g)	199.63 ± 6.90^{a}	220.24 ± 6.46^{b}	140.32 ± 3.62^{c}	151.75 ± 3.20^{d}
Pelt BF%	80.27 ± 5.14^{a}	62.51 ± 2.50^{b}	93.10 ± 2.43^{c}	94.75 ± 1.46^{c}
Pelt Fat (g)	44.80 ± 1.98^{a}	61.27 ± 4.07^{b}	37.43 ± 1.69^{c}	38.43 ± 2.35^{ac}
Pelt Fluid (g)	40.21 ± 2.36^{a}	61.51 ± 3.47^{b}	26.33 ± 0.53^{c}	27.61 ± 1.09^{c}

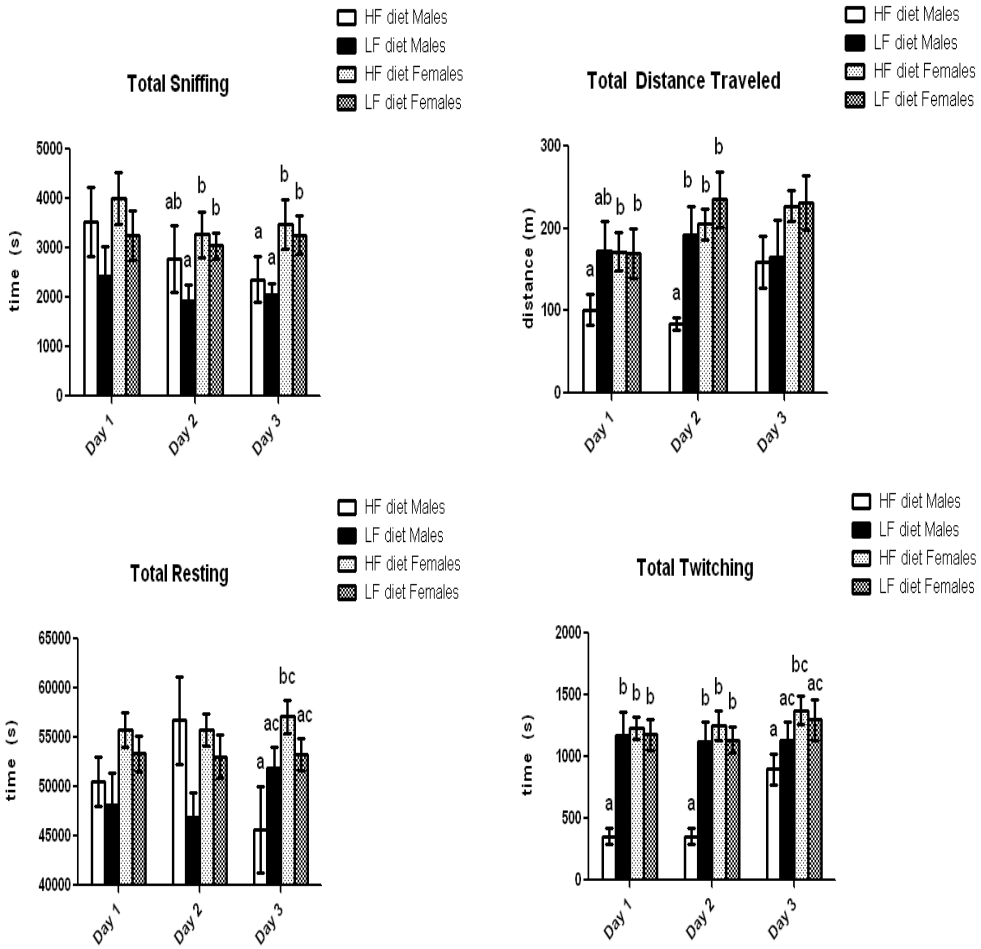
Legend: Food intake (FI), body weight (BW), body fat (BF), lean body mass (LBM). Statistics represent differences across rows. Results with different letters differ at $p< 0.05$.

Food Intake

In males and females, rats on the HF diet consumed more calories in 72 hours than the rats on the LF diet. No difference was observed between males and females on the HF diet; however LF diet females consumed fewer calories than males on the LF diet.

Body Weight

Females fed a HF diet had a larger increase in body weight compared to their LF diet counterparts, whereas no difference was observed between the male diet groups. Additionally, no differences were observed between sexes within each diet groups.



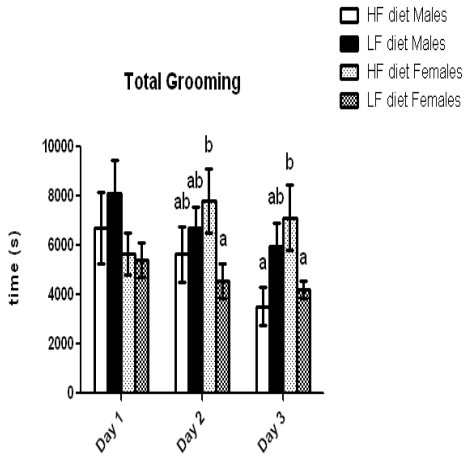


Figure 3-1. **HomeCage Scan Behaviors-** changes in behavior during 72 hours of HF diet assessed by HomeCage Scan. Data include total distance traveled; total time spent sniffing, resting, and twitching. Statistics represent differences observed between groups on each day. Results with different letters differ at $p<0.05$.

Cage Activity

Males on a HF diet traveled less on the second day of the diet compared to males on a LF diet. There were no differences between female diet groups. Females on the HF diet traveled farther on days 1 and day 2 of the experiment compared to HF diet-fed males. There were no differences between the LF groups.

Females on the HF diet groomed more on day 2 of the diet compared to their LF diet counterparts. No sex differences were observed between the LF groups, yet females on the HF diet groomed more than the males on day 3.

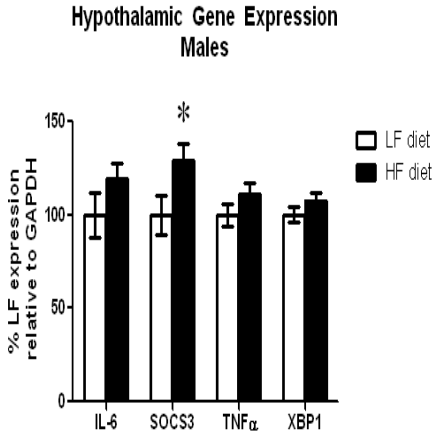
No diet differences were observed in the amount of time spent sniffing. However, females on the HF diet sniffed more than HF diet-fed males on day 3. LF diet females also sniffed more days 2 and 3 compared to males.

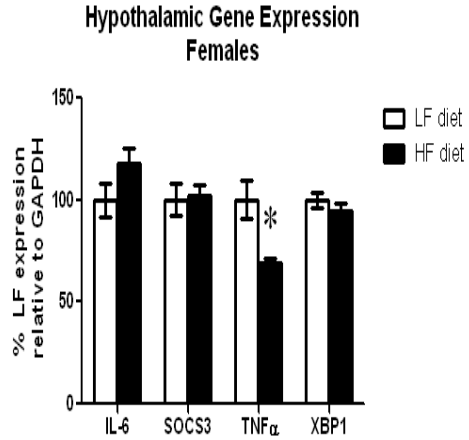
Females on the HF diet rested more on day 3 compared to HF diet males. Twitching is a behavior observed during sleep and is quantified by quick moments of activity between bouts of sleeping. While no difference was observed between HF and LF diet-fed females, males on the HF diet twitched less compared to their LF diet counterparts. These differences were observed on day 1 and day 2. Males on the HF diet twitched less than females on the HF diet each day.

Body Composition

Pelt represents subcutaneous fat whereas the carcass contains the muscle of the body wall encasing the visceral fat. There were no diet effects in females; however males on the HF diet had more fat in the body and the pelt compared to LF males. By weight, males on a HF diet had more fat and lean body mass than females on a HF diet. However, by percentage HF diet males had lower body fat percentages in both the body and pelt compared to females on a HF diet. In the LF diet groups, females again had increased body fat percentages in the body and pelt, whereas males had increased lean body mass.

Inflammation





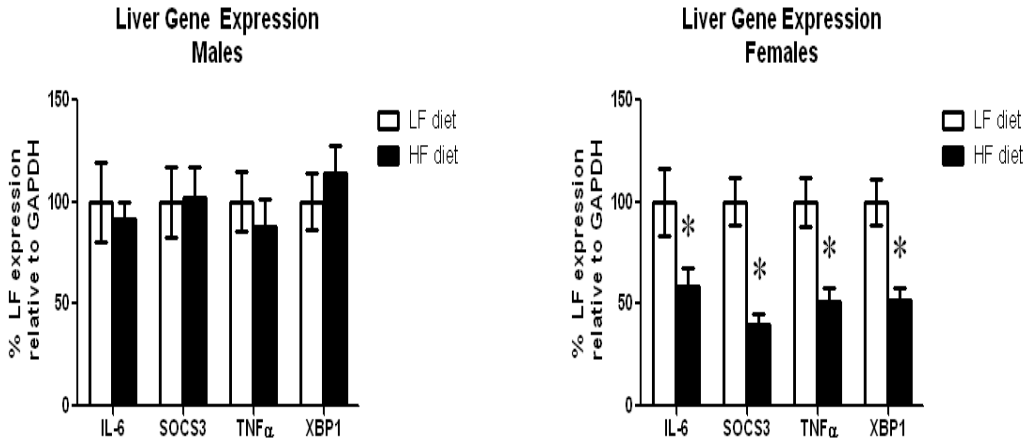


Figure 3-2. **Effect of Diet on Inflammation-** Changes in gene expression after 72 hours on the HF diet assessed by qPCR. Data include inflammatory gene expression (IL-6, SOCS3, TNFα) and ER stress (XBP1). Statistics represent differences observed between diet groups at each gene. Results with stars differ at $p<0.05$.

Effect of Diet on Inflammation

In the hypothalamus, the only difference found in male rats was that males on the LF diet had lower expression of SOCS3 than males on the HF diet. Additionally, females on the HF diet had lower expression of TNFα than their LF diet counterparts. There were no differences in IL-6. In the liver, females on the HF diet had lower expression of IL-6, SOCS3, and TNFα than females on the LF diet. No diet differences were observed amongst males.

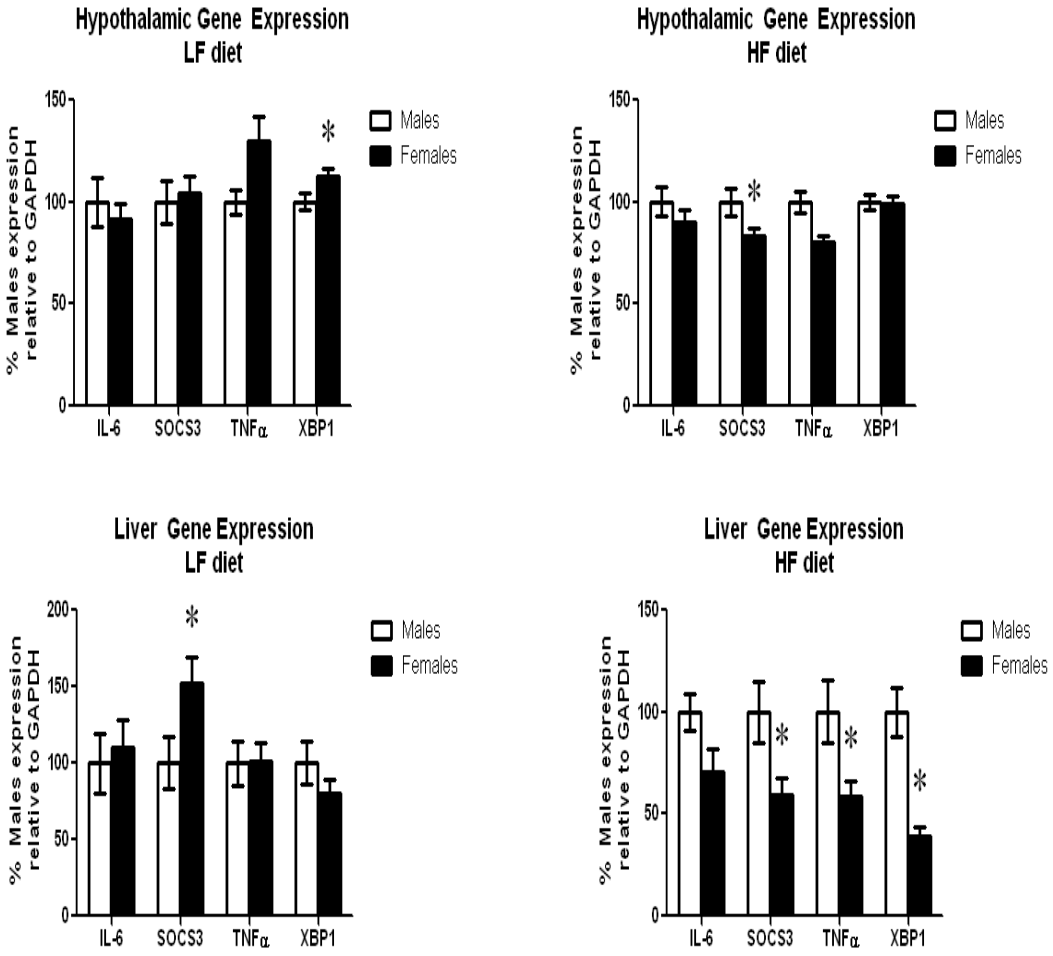


Figure 3-3. **Effect of Sex on Inflammation-** changes in gene expression after 72 hours of HF diet assessed by qPCR. Data include inflammatory gene expression (IL-6, SOCS3, TNFα) and ER stress (XBP1). Statistics represent differences observed between sexes at each gene. Results with stars differ at $p<0.05$.

Effect of Sex on Inflammation

In the hypothalamus, HF diet females had lower expression of SOCS3 than males. There were no differences in IL-6 in the hypothalamus. In the liver, HF diet females had lower expression of SOCS3 and TNFα than males. Additionally, LF diet males had lower expression of SOCS3 than females. Once again, there were no differences in IL-6.

ER Stress

Effect of Diet on Marker of ER Stress

Endoplasmic reticulum stress was measured by expression of XBP1. Increased expression of this protein reflects increased ER stress. No dietary differences in XBP1 expression were found in either sex. In the liver, females on the HF diet had lower expression of XBP1 than females on the LF diet.

Effect of Sex on Marker of ER Stress

In the hypothalamus, males on the LF diet had lower expression of XBP1 than females on the LF diet. There were no differences in hypothalamic XBP1 in the HF diet groups. In the liver, females on the HF diet had lower expression of XBP1 than males on the HF diet. There were no differences in XBP1 expression in the liver between the LF groups.

Discussion

In this study we demonstrated differing effects of endogenous estrogen on inflammatory gene expression. Overall, females displayed a dramatic ability to prevent HF diet-induced inflammatory stress compared to males during short-term exposures to HF diet. The hypothalamus is an important regulator of energy balance and its disruption, which can be caused by HF diet, may result in obesity and diabetes in rats (Zhang et al., 2008).

In our study, male rats on a HF diet had increased expression of SOCS3 in the hypothalamus compared to LF controls. Increased SOCS3 signaling in the hypothalamus can directly result in both central leptin and insulin resistance (Howard et al., 2004; Mori

et al., 2004; Zhang et al., 2008). Intracellular SOCS3 is able to prevent JAK phosphorylation on the leptin receptor and interrupting central leptin signaling (Bjorbaek et al., 1999). Previous studies have determined that central leptin and insulin resistance occurs in male rats after 72 hours of a HF diet (Wang et al., 2001; Morgan et al., 2004). The increased SOCS3 expression found in the current study may be a mechanism for hypothalamic leptin and insulin resistance when rats are given a HF diet.

When adjusting for sex, males on a HF diet also had increased hypothalamic expression of SOCS3 compared to females on a HF diet. This was not surprising because females often maintain central leptin and insulin sensitivity when fed a HF diet, and are less susceptible to DIO (Clegg et al., 2006; Shi and Clegg, 2009). This was reflected in our study since females given a HF diet had no change in adiposity, but males increased body fat in their pelt and body. We therefore suggest that in our study females may have been protected from central leptin and insulin resistance due to reduced hypothalamic inflammation though this was not measured. Additionally, the reduced inflammatory expression observed in females may provide a mechanism for the protection from HF diet-induced increases in adiposity.

In female rats, HF diet resulted in reduced inflammatory expression in the hypothalamus and prevented the increase in SOCS3 expression observed in males. However, there was no change in IL-6 expression. IL-6 is an anti-obesogenic cytokine that plays a protective role in maintaining central insulin and leptin sensitivity (Sadagurski et al.; Wallenius et al., 2002; Flores et al., 2006). Intracerebral 3rd-ventricle IL-6 treatment in IL-6 knockout mice increased energy expenditure and reduced food

intake, and prevented obesity (Wallenius et al., 2002). Taking this into account, an additional way female rats may be protected from central leptin and insulin resistance may by maintaining IL-6 expression in the hypothalamus.

ER stress plays a major role in central leptin resistance, obesity, and insulin resistance (Ozcan et al., 2004; Zhang et al., 2008; Ozcan et al., 2009). Accumulation of unfolded proteins in the ER lumen occurs during the rapid cellular growth that occurs with overnutrition and increased inflammation. Additionally, saturated free fatty acids cause ER stress by interacting with TLR4 (Milanski et al., 2009). Unfolded proteins activate IRE-1 and the unconditional splicing of the transcription factor XBP1. Activation of IRE-1 also results in activation of inflammatory signaling cascades including NFκB and JNK (Zhang and Kaufman, 2008). Through these mechanisms, it is suggested that ER stress plays an important role in both leptin and insulin resistance.

Interestingly, we observed an increased expression of XBP1 in the liver of the HF diet males compared to their female counterparts. In the liver, XBP1 has functions outside of ER stress and is a major regulator of *de novo* lipogenesis (Lee et al., 2008). Increases in blood glucose results in the expression and activation of XBP1 which leads to dyslipidemia and increased fat deposition in adipocyte stores (Glimcher and Lee, 2009). Taking this into account, it is plausible that the increased adiposity observed in HF diet-fed males may have been due to XBP1-driven lipogenesis. This finding may provide a novel explanation on increased metabolic disease in males.

Both sexes on the HF diet consumed more energy than those on the LF diet. This is conflicting to what others have observed on 72 hours of similar diets. Some have

observed no differences between HF and LF-fed male rats after 3 days of feeding (Naderali and Williams, 2001; Wang et al., 2001; Morgan et al., 2004). However, others have observed differences in energy intake after 72 hours (Wang et al., 2001; Morgan et al., 2004). In some cases rats fed a HF diet for 7 days or less are not hyperphagic. To our knowledge, we are the first to observe hyperphagia in females given a HF diet for 72 hours. Results from this study suggest that both sexes are unable to adjust their energy consumption to the increased caloric content of a HF diet during the first 72 hours of administration.

Interestingly, HF females did not display increased fat deposition in either pelt or body tissue. Males given the HF diet increased fat mass in their carcass and pelt. Therefore, while HF females may have eaten more and gained more weight, they did not deposit this as fat. The measured weight in females was water. This suggests that females may somewhat be protected against increased adiposity caused by short exposures to HF diets by potentially increasing activity and energy expenditure since there was no decrease in caloric consumption.

In conclusion, data obtained from our study suggest that females may be protected more from diet induced inflammation than males. In the hypothalamus HF diet-fed females maintained IL-6 expression while reducing TNFα expression. Conversely, HF diet in males increased inflammatory expression (SOCS3). Additionally, there was a decrease in hepatic inflammatory expression (IL-6, SOCS3, TNFα) in HF diet-fed females. Therefore we present a possible explanation for reduced inflammatory disease in

females and suggest a potential area for further investigation of the role of E2 in mediating central and peripheral responses to a HF diet.

Acknowledgements

We thank Ken Gruber for assistance with the statistical analyses. Additionally we thank Stephen Benoit, Robin Hopkins, Keshia Ramseur, and Tricia Kauffman for technical assistance.

CHAPTER IV

ESTRADIOL PREVENTS INCREASES IN HYPOTHALAMIC SOCS3 EXPRESSION AFTER 24 HOURS ON A HIGH-FAT DIET

Abstract

There is evidence that obesity is accompanied by chronic activation of inflammatory pathways. Ovarian hormones including estradiol (E2) and progesterone have anti-inflammatory effects in the hypothalamus and liver. In the present study we sought to determine if this effect is present when a high-fat (HF) diet is first introduced. E2 or vehicle treated ovariectomized (OVX) Long-Evans rats (three-months old) were given a HF or a low-fat (LF) diet for 24 or 72 hours (n= 80). A subset of each diet group received either 2 μg E2 or vehicle every 4 days. The liver and hypothalamus were extracted and mRNA concentrations were determined using quantitative PCR of IL-6, SOCS3, TNFα, and XBP1. E2 replacement in the HF diet-fed rats prevented increases in SOCS3 expression after 24 hours of a HF diet in the hypothalamus. Additionally, no changes in IL-6 occurred after 24 hours of a HF diet in the hypothalamus. Endoplasmic reticulum (ER) stress, measured by higher expression of XBP1, was increased in vehicle-treated rats given a HF diet compared to LF controls. These results suggest that cyclic E2 replacement prevents HF diet-induced increases in hypothalamic stress during the first 24 hours of a HF diet. These findings may provide support for the protective role of E2 in HF-induced inflammation.

Introduction

Obesity is the second leading cause of preventable death in the United States (Lehrke and Lazar, 2004; Hotamisligil, 2006). Its development increases the risk for type 2 diabetes, cardiovascular disease, certain cancers, and neurodegenerative disorders. Men and postmenopausal women have a greater risk of developing obesity and the metabolic syndrome than premenopausal women (Shi and Clegg, 2009). This suggests that ovarian hormones (estrogen and progesterone) may interact with cellular pathways in the central nervous system and the periphery to protect women from diet-induced obesity (DIO) and increased adiposity (Brown and Clegg, 2009).

Young female rats often do not become obese when fed a HF diet, whereas aged-matched males will (Shi and Clegg, 2009). After ovariectomy (OVX), rats lose this protection and gain weight at a level significantly higher than intact female rats (Shimizu et al., 1996). E2 replacement in OVX rats reverses this, restoring body weight to that of intact rats (Asarian and Geary, 2002). Interestingly, we observe a similar occurrence in women on hormone replacement therapy (Haarbo et al., 1991). While it is important to note that there may be a heightened risk for estrogen-sensitive breast cancer (Rossouw et al., 2002), hormone replacement therapy in postmenopausal women often results in a shift to reduce visceral fat deposition and reduced risk for the metabolic syndrome (Haarbo et al., 1991; Colacurci et al., 1998).

Inflammation plays an important role in the development of obesity (Hotamisligil, 2006; Yang and Hotamisligil, 2008). Inflammatory signaling in the hypothalamus can

directly result in central insulin and leptin resistance (Yang and Hotamisligil, 2008). Saturated fat in particular can activate toll like receptors in the hypothalamus causing the activation of NFκB signaling cascades (Milanski et al., 2009). In addition, consumption of HF diets has shown to result in the expression of NFκB responsive genes including SOCS3 and TNFα. SOCS3 signaling inhibits Ob-Rb phosphorylation and activation of the JAK-STAT signaling cascade (Bjorbaek et al., 1999; Zhang et al., 2008). Suppression of SOCS3 in the hypothalamus results in enhanced leptin sensitivity and an inability to gain weight on obesogenic diets (Mori et al., 2004).

Interestingly, females often display increased leptin sensitivity compared to males on LF diets (Shi and Clegg, 2009). A potential mechanism by which female rats may be protected from DIO is through maintaining central sensitivity to the leptin and insulin (Brown and Clegg, 2009). Additionally, E2 is a potent anti-inflammatory hormone in the central nervous system (Straub, 2007). It has a wide range of intracellular effects including upregulation of free radical scavengers and disrupting NFκB signaling (Ghisletti et al., 2005; Straub, 2007; Stice and Knowlton, 2008). Reduced activation of NFκB represses expression of pro-inflammatory cytokines, a possible mechanism by which females display reduced weight gain on HF diets.

In the present study we sought to further investigate the role of E2 in reducing inflammatory gene expression and preventing an increase in body fat when females are given a short exposure to HF diet (72 hours). In a previous study we observed that females in proestrus had reduced inflammation in the hypothalamus and liver after 72

hours on a HF diet compared to their LF-fed controls. Additionally, hypothalamic SOCS3 expression was increased in males given a HF diet, whereas females maintained SOCS3 expression when given a HF diet. Therefore the primary aim of this study was to assess inflammatory gene expression in both the liver and the hypothalamus in OVX rats given a 4 day cyclic E2 replacement schedule. We also investigated both behavioral changes in food intake and activity, in addition to changes in body composition and weight.

Materials and Methods

Animals

OVX (n=80) Long-Evans rats were purchased from Harlan Labs (Harlan Labs; Indianapolis, IN). Upon arrival they were given 1 week to acclimate to the facility before introduction to colony rooms. 6 days post-surgery the surgical staples were removed under isofluorane anesthesia and triple antibiotic was applied to the incision site. Prior to the start of the experiment rats received phytoestrogen-free chow (11% fat and 3.1 kcal/g, #2014, Harlan Teklad; Indianapolis, IN) to reduce the effects of environmental estrogens on the results (Table 1). Rats had free access to food and water *ad libitum* throughout the experiment. Rooms were temperature (22 $\pm$ 2 °C) and humidity controlled and kept on a 12:12 light/dark cycle (lights on at 4 am). At the start of the experiment, a subset of each group was switched to a HF diet (40% fat and 4.54 kcal/g, #D03082706, Research Diets; New Brunswick, NJ) (Table 4-1) while the others remained on chow. This HF diet uses butter as the fat source. It was selected to match the major source of fat in the US diet. Rats and their food were weighed daily. The University of North Carolina at Greensboro Institutional Animal Care and Use Committee approved all protocols for this experiment.

Table 4-1. **Diet Composition**

Dietary Components	Phytoestrogen-free Chow	HF Diet
	% total kcal	% total kcal
Carbohydrate	71%	45%
Protein	18%	15%
Fat	11%	40%
Total kcal/g	3.1	4.54

Estradiol Replacement

2.0 µg 17 β-estradiol-3-benzoate (Fisher Scientific; Pittsburgh, PA) was dissolved in 100 % ethanol and added to sesame oil (2.0 µg /100 µl) and injected every 4 days after OVX (Asarian and Geary, 2002). The injection paradigm was designed so that the final injection would occur within 18 hours prior to sacrifice. Half of the OVX rats received E2 replacement. Control groups received injections of the sesame oil vehicle on the same schedule.

Behavioral Testing

Measurements of home cage behaviors were performed through real-time video surveillance and HomeCage Scan software (Clever Systems, Inc; Reston, VA). The room was set up with a dark blue background and a red light under each cage for monitoring movement of each animal during the night cycle. Animals were given a 1 day acclimation period to the behavioral room with the video cameras running prior to the start of the 72 hour study. Variables recorded included ambulatory behaviors, exploratory behaviors, rearing behaviors, eating, drinking, and sleeping in both seconds and number of bouts.

Plasma Analysis

After 72 hours on their respective diets the rats were euthanized by decapitation. Trunk blood was collected in heparinized tubes and centrifuged. Aliqouts of plasma were collected and stored at –80 °C until analyzed. Plasma leptin was measured using a rat leptin radioimmunoassay (RIA) kit (Linco Research, St. Charles, Missouri). This assay is able to detect leptin in 100 µl samples of plasma. Plasma insulin will be measured by ELISA using a Labsystems Multiscan Plus plate reader (Fisher Scientific; Pittsburgh, PA). Serum concentrations of estradiol will be measured by specific radioimmunoassay (Quest Diagnostics, Inc.-Nichols Institute Diagnostics, San Juan Capistrano, CA).

Inflammatory Gene Expression

At sacrifice, the medial basal hypothalamus and a section of liver were 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). 2ng 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 quantitative PCR using Applied Biosystems primers for TNFα, SOCS3, IL-6, and XBP-1 (Table 4-2).

Table 4-2. **Applied Biosystems Primers for qPCR**

PRIMERS	Product Number
TNFα	Rn1525860_g1
SOCS3	Rn00585674_s1
IL-6	Rn99999011_m1
XBP1	Rn01752569_m1
GAPDH	Rn01462662_g1

Legend: primers were purchased from Applied Biosystems (Foster City, CA).

Body Composition

After sacrifice, the skin and subcutaneous fat (pelt) was dissected from the muscle wall and visceral fat (carcass) so that subcutaneous fat and visceral fat could be measured. The separated parts were frozen at -20 °C until measurement by Dual Energy X-Ray Absorptiometry scan. Both the pelt and body were scanned in duplicate to determine % body fat and % lean body mass.

Statistical Analysis

Statistical analyses were performed using SPSS (version 17.0). To analyze specific planned comparisons involving diet conditions and sex groups, independent t-tests were performed. Significance was set at $p<0.05$; data results are presented as means with corresponding SEMs.

Results

Table 4-3. **72 Hour Group Food Intake, Body Weight and Composition**

	LF diet Vehicle	HF diet Vehicle	LF diet E2	HF diet E2
72 hr FI (kcal)	241.85 ± 5.67 [a]	392.05 ± 15.87 [b]	218.40 ± 6.76 [c]	349.43 ± 14.98 [b]
72 hr BWΔ (g)	9.22 ± 1.74 [a]	16.03 ± 1.67 [b]	2.02 ± 0.79 [c]	15.79 ± 1.07 [b]
Body BF%	21.25 ± 0.70	26.19 ± 0.78	23.01 ± 0.87	25.22 ± 0.84
Body Fat (g)	43.17 ± 1.63 [a]	56.67 ± 2.63 [b]	42.64 ± 2.14 [a]	50.89 ± 2.21 [abc]
LBM (g)	160.00 ± 3.39 [a]	159.78 ± 2.85 [a]	141.82 ± 2.06 [b]	151.22 ± 2.65 [c]
Pelt Fat (g)	28.92 ± 1.18 [a]	35.89 ± 1.91 [b]	23.45 ± 2.13 [c]	32.11 ± 2.72 [ab]

Legend: Food intake (FI), body weight (BW), body fat (BF), lean body mass (LBM). Statistics represent differences across rows. Results with different letters differ at $p < 0.05$.

Table 4-4. **24 Hour Group Food Intake, Body Weight and Composition**

	LF diet Vehicle	HF diet Vehicle	LF diet E2	HF diet E2
24 hr FI (kcal)	73.94 ± 4.24 [a]	137.31 ± 6.62 [b]	72.79 ± 1.38 [a]	126.11 ± 4.23 [b]
24 hr BWΔ (g)	1.62 ± 1.80 [a]	8.03 ± 1.27 [b]	0.23 ± 0.80 [a]	6.03 ± 0.98 [b]
Body BF%	23.17 ± 0.87	22.92 ± 0.95	39.06 ± 16.21	24.11 ± 0.85
Body Fat (g)	45.91 ± 2.16 [a]	48.67 ± 2.36 [a]	41.00 ± 2.10 [ab]	47.89 ± 2.06 [ac]
LBM (g)	151.36 ± 4.41 [a]	162.89 ± 3.44 [a]	139.36 ± 2.66 [b]	150.22 ± 2.87 [ac]
Pelt Fat (g)	26.45 ± 1.74 [a]	27.89 ± 1.87 [a]	20.09 ± 1.26 [b]	26.56 ± 2.25 [a]

Legend: Food intake (FI), body weight (BW), body fat (BF), lean body mass (LBM). Statistics represent differences across rows. Results with different letters differ at $p < 0.05$.

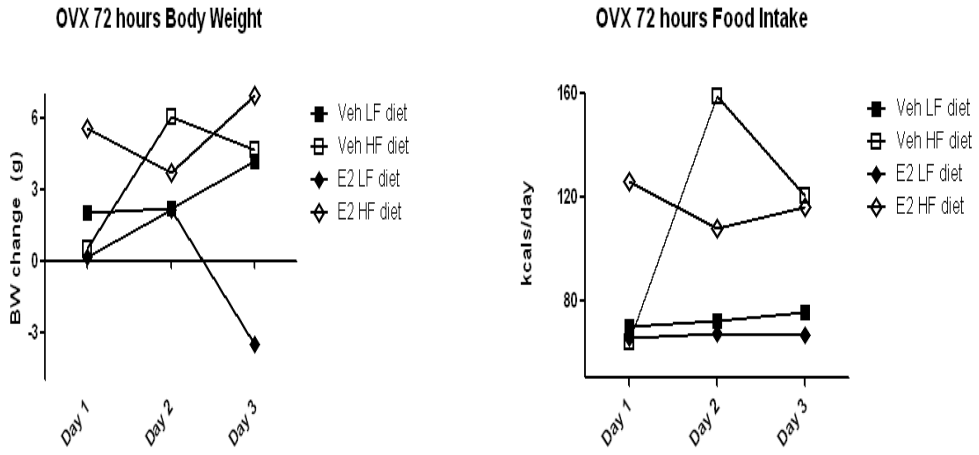


Figure 4-1. **72 hour Food Intake and Body Weight Change-** food intake and body weight changes during 72 hours of HF diet assessed by daily weighing.

Food Intake and Body Weight

72 Hour Sacrifice

E2 replacement resulted in reduced caloric intake and body weight gain over 72 hours in the LF diet group compared to vehicle-injected controls, however this was not observed in the HF group. Additionally, HF fed rats of both treatments increased their food intake and body weight compared to their LF counterparts.

24 Hour Sacrifice

In the 24 hour experiment, HF fed rats of both treatments had an increased caloric intake and body weight gain compared to LF counterparts. No additional differences were observed between groups.

Body Composition

72 Hour Sacrifice

The total fat of the carcass and pelt was significantly increased in both HF groups compared to their LF counterparts after 72 hours. Additionally, within the LF groups, pelt fat was increased in the vehicle-treated rats compared to E2-treated rats. HF diet in the E2 treated group resulted in an increase in lean body mass of the carcass compared to the LF control. Additionally, both vehicle treated groups had an increased lean body mass compared to their E2 treated counterparts.

24 Hour Sacrifice

In the 24 hour experiment, E2 treated rats on a HF diet had more carcass and pelt fat compared to their LF-fed controls. Additionally, pelt fat was reduced in the E2-treated, LF rats compared their vehicle- treated counterparts. Lastly, LF and HF vehicle-treated groups had an increase in lean body mass compared to E2 treated animals. However, an increase in lean body mass was observed in the HF E2-treated rats compared to their LF controls.

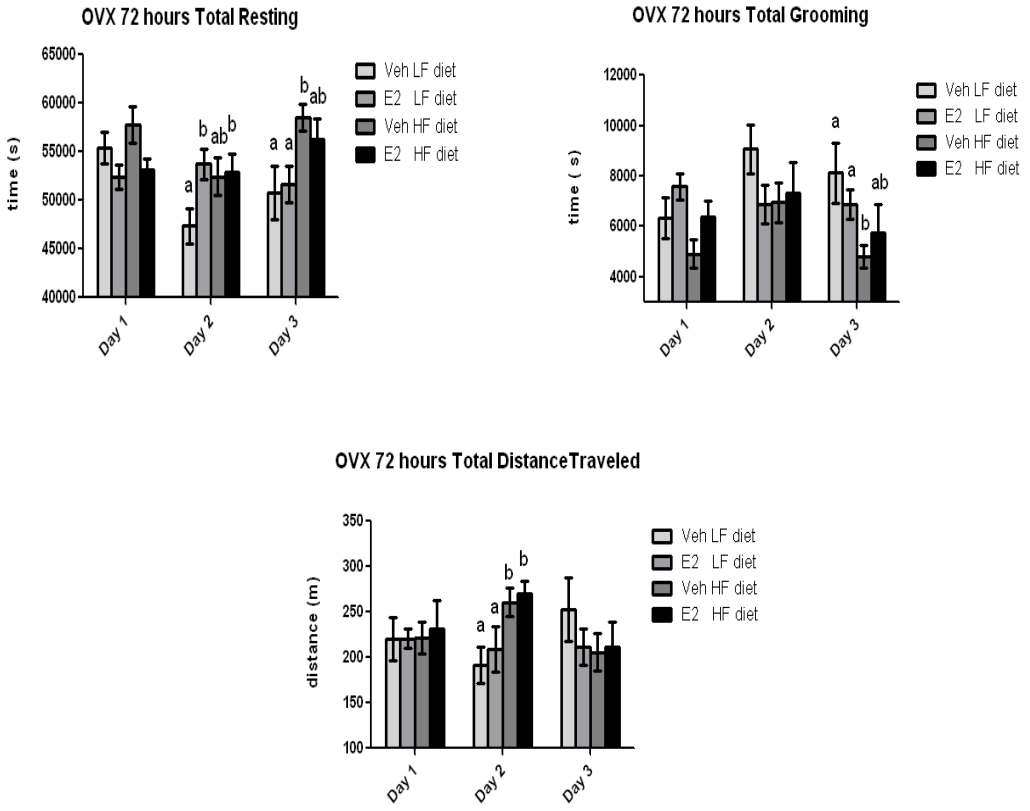


Figure 4-2.**HomeCage Scan Behaviors-** changes in behavior during 72 hours of HF diet assessed by HomeCage Scan. Data include total time spent resting and grooming, and total distance traveled. Ovariectomized (OVX); vehicle treatment (Veh); estradiol treatment (E2). Statistics represent differences observed between groups on each day. Results with different letters differ at $p<0.05$.

Cage Activity

72 Hour Sacrifice

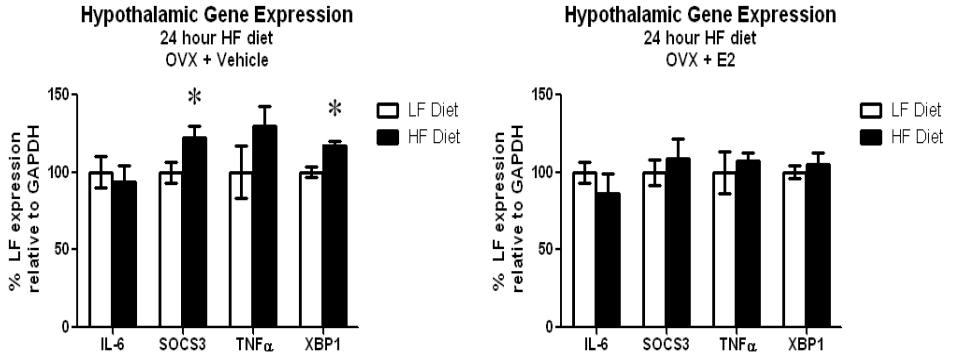
In the 72 hour study, we did not observe any differences in any behaviors between the LF and HF diet E2 groups. However, in the vehicle group we observed a reduction in grooming on day 3 and an increase in total distance traveled on day 2 in the HF-fed rats

compared to their LF controls. Within resting, we observed an increase in the amount of time spent resting on day 3 in the HF vehicle group compared to their LF control. Additionally, within the LF groups, rats given E2 replacement had an increase in resting on day 2 compared to vehicles.

24 Hour Sacrifice

In the 24 hour study, there was a significant reduction in grooming in E2 treated rats on a HF diet compared to their LF counterparts. Additionally, in the LF groups, E2 treated rats spent more time grooming than vehicle-treated controls. Within the LF diet groups, E2 treated rats displayed increased time sniffing but reduced time twitching compared to vehicle treated controls (data is not shown).

Inflammatory Gene Expression



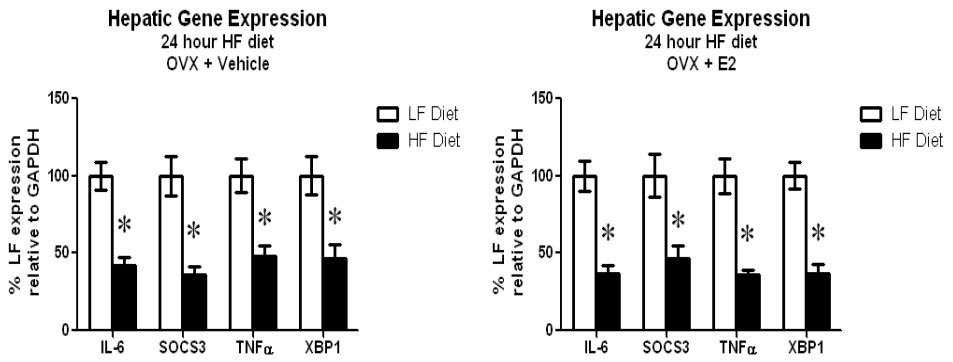


Figure 4-3. **Gene Expression after 24 Hours on a HF Diet-** Data include inflammatory gene expression (IL-6, SOCS3, TNFα) and ER stress (XBP1). Statistics represent differences observed between diet groups. Results with stars differ at $p<0.05$.

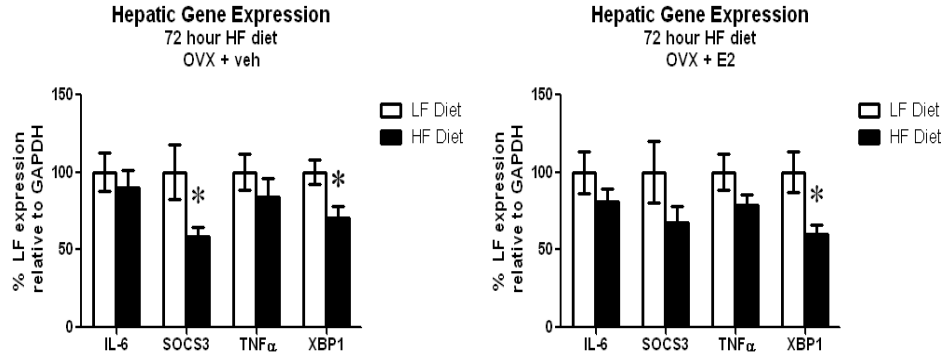


Figure 4-4. **Gene Expression after 72 Hours on a HF Diet-** Data include inflammatory gene expression (IL-6, SOCS3, TNFα) and ER stress (XBP1). Statistics represent differences observed between diet groups. Results with stars differ at $p<0.05$.

Effects of Diet on Inflammatory Gene Expression

In the hypothalamus, vehicle-treated rats on the LF diet had lower expression of SOCS3 than those on the HF diet. No other differences were observed. In the liver, OVX rats given the two diets for 24 hours had the same changes for all genes measured. Vehicle treated rats on the HF diet had lower expression of IL-6, SOCS3, and TNFα than their LF controls. Additionally, E2 treated rats on the HF diet had lower expression of IL-6, SOCS3, and TNFα compared to their LF controls. OVX rats given the two diets for 72 hours also had similarities. Vehicle-treated rats on the HF diet for 72 hours had lower expression of SOCS3 than their LF controls.

ER Stress

Effects of Diet on Marker of ER Stress

In the hypothalamus, vehicle-treated rats on the LF diet had lower expression of XBP1 than those on the HF diet for 24 hours. No other differences were measured. In the liver, OVX rats given the HF diet for 24 hours had lower expression of XBP1 than their LF controls. OVX rats given the two diets for 72 hours also had similarities. Within both treatment groups, OVX rats given the HF diet for 72 hours had reduced XBP1 expression compared to their LF controls in the liver.

Discussion

E2 has shown to be anti-inflammatory in various disease models in rats (Jansson and Holmdahl, 1998; Ghisletti et al., 2005; Pozzi et al., 2006). Its protective properties are often suggested to be due its many interactions with the NFκB complex (Ghisletti et al., 2005). For this reason we chose to investigate three NFκB responsive genes (SOCS3,

IL-6, TNFα). In this study we found that E2's effects on inflammatory gene expression after short exposures to HF diet varies widely by tissue.

Much like what is observed in female rats in proestrus; our OVX model was protected from HF diet-induced increases in hypothalamic SOCS3 expression when given cyclic E2 replacement. Additionally, our rats also maintained IL-6 expression in the hypothalamus at both time points. Leptin signaling in the hypothalamus has been shown previously to be dependent on IL-6 mediated pathways (Sadagurski et al.; Wallenius et al., 2002; Flores et al., 2006). Removing IL-6 in the hypothalamus promotes weight gain and obesity through increased energy consumption and reduced energy expenditure (Wallenius et al., 2002). E2 has previously been shown to be protective in the hypothalamus by enhancing leptin sensitivity, particularly by increasing the availability of Ob-Rb (Bennett et al., 1998). Additionally, intact females and E2 replacement in OVX-models show a protection from DIO (Shi and Clegg, 2009). E2 exposure in hypothalamic cells has shown recently to increase expression of IL-6 (Ogura et al., 2008), though to our knowledge no one has investigated this response in terms of central leptin and insulin sensitivity. While we did not observe an increase after HF diet, but we did not see a reduction in IL-6 expression. We therefore suggest that a potential mechanism E2 is enhancing hypothalamic leptin sensitivity is by maintaining IL-6 expression when under diet-induced stress.

Although we did not determine central leptin or insulin sensitivity, we did measure changes in food intake, body weight, spontaneous physical activity, and body composition. Much like what we observed in intact females, E2 treatment provided no

protection from HF diet-induced increases in food intake and weight gain over the 72 hours of the study. In a previous study looking at rats in proestrus, the increased body weight was neither in lean body mass nor body fat. Using NMR body composition data we were able to determine that the increase in body weight was due to increased water weight. E2-treated rats did not show any protection to changes in body composition caused by short exposures to HF diet and displayed similar changes in body fat as vehicle-treated rats.

In summary, in our experiment E2 replacement did little to protect OVX rats from the adverse effects of short exposures to HF diets on body weight and fat. Systemic inflammation is a characteristic of the etiology of obesity. While rats given E2 replacement did not increase inflammatory gene expression at any time point in any of the tissues measured, there were no differences observed in our vehicle-treated controls. Interestingly, after 24 hours on a HF diet both treatment groups had marked reductions in inflammatory gene expression in the liver. However gene expression does not allow us to determine protein levels, which could be increased.

A potential reason for the conflicting results we obtained could have been due to the time that was given for recovery after the OVX surgery. It is possible that our animals may have had increased inflammation because they had surgeries. If there was a systemic inflammation that had occurred that was independent of the diet, it would be difficult to determine differences caused by E2 treatment and/or HF diet.

However, while E2 replacement did not provide protection from the increased inflammatory gene expression that results from a HF diet, we did see a protection from

HF-diet induced increases in hypothalamic SOCS3 expression that was observed in males fed a HF diet for 24 hours. Additionally, E2 treatment also resulted in maintenance of hypothalamic IL-6 expression during the first 24 hours of a HF diet. We therefore provide a potential novel mechanism for enhanced leptin sensitivity normally observed in female rats given a HF diet. Estrogenic compounds that can reduce intracellular SOCS3 while enhancing IL-6 signaling selectively in the central nervous system may provide potential therapeutic targets for the treatment of obesity.

Acknowledgements

We thank Ken Gruber for assistance with the statistical analyses. Additionally we thank Stephen Benoit, Robin Hopkins, Keshia Ramseur, and Tricia Kauffman for technical assistance.

CHAPTER V

EPILOGUE

Overall Conclusions

In these studies we determined that circulating E2 might protect females from HF diet-induced inflammation that may in turn result in central leptin and insulin resistance. In proestrus females we observed reductions in TNFα expression and protection from HF diet-induced increases in SOCS3 expression observed in males. OVX + veh rats were similar to males in that they increased SOCS3 expression after 24 hours of a HF diet, whereas OVX + E2 were similar to intact females after since they did not increase SOCS3 expression in the hypothalamus. Thus, these findings suggest that E2 can manipulate gene expression that may allow female rats to retain sensitivity to anorexogenic hormones in the hypothalamus under HF diet-induced stress.

Increased SOCS3 in the hypothalamus directly results in blunted insulin and leptin signaling (Zhang et al., 2008). SOCS3 is able to interact with receptor substrates preventing downstream signaling once insulin and leptin bind to their ligand (Bjorbaek et al., 1999). Therefore the increased SOCS3 observed in HF diet-fed males might correlate to central leptin and insulin resistance after 72 hours of HF diet observed in previous studies.

In addition to preventing increased SOCS3 expression, intact females were also able to retain normal IL-6 expression levels. IL-6 is an important component of leptin and insulin signaling in the hypothalamus (Sadagurski et al.; Wallenius et al., 2002; Flores et al., 2006). IL-6 knock-out mice become obese and have central leptin and insulin resistance (Wallenius et al., 2002). In our studies, both the intact females and OVX + E2 rats were able to retain IL-6 expression in the hypothalamus compared to LF diet controls. While both groups did increase their 72 hour food intake and body weight compared to LF diet controls, the intact females did not increase body fat. Central inflammation may have been controlled, and this may have allowed rats to retain central leptin and insulin sensitivity. While central leptin and insulin resistance was not directly measured in our studies, it would be a useful component of future studies.

Lastly, in addition to a significant reduction in inflammatory expression in the liver, we found a sex difference in XBP1 expression in the liver. HF diet-fed males had an increased expression of hepatic XBP1 compared to their female counterparts. In the liver, XBP1 has an ER stress-independent function of regulating *de novo* lipogenesis (Lee et al., 2008; Glimcher and Lee, 2009). Males have increased lipogenic activity within the liver compared to females (Born et al., 2003); a potential reason for this could be increased XBP1 expression. Additionally, increased lipogenic activity in the liver might also help explain the increased adiposity in our males fed a HF diet. Therefore we suggest that males have increased hepatic XBP1 expression in an ER stress-independent manner which might help explain the increased susceptibility for male rats to become obese on HF diets.

Study Difficulties

We used a novel program to determine changes in behavior due to HF diet; therefore it is difficult to compare our results with previous literature. In addition, we ran into various problems while measuring and obtaining data. The largest problem that occurred was with the color of our animals. HomeCage Scan was developed to view animals that are either dark or light colored. Because Long-Evans rats are hooded black and white rats, the program had settings that would only pick up white or black and missed a portion of the rat. In order for the program to see the entire rat, we were forced to lose sensitivity of the recording. While other investigators who used the HomeCage Scan report over 90% accuracy (Steele et al., 2007), I do not believe we obtained that level of accuracy. Due to this, if this study were to be repeated, I would suggest using behavioral equipment to measure stress or anxiety like the Open-Field tests or Elevated Plus Maze. It is important to note that we were in constant contact with the developers of the program throughout the experiment and twice the software engineer visited campus to assist us with our problems. Several program updates were written to address our issues and we were able to collect behavioral data in our studies.

The primary aim of this thesis was to determine how E2 effects inflammatory gene expression caused by short-term administrations of a HF diet. To assess this, animals were sacrificed at the point where plasma E2 would be its highest. This increase is observed during proestrus in intact females and 18 hours after the E2 injection in the OVX model (Asarian and Geary, 2002). Because the changes in behavior caused by increased E2 occurs in the dark cycle of estrus (day 4), the timing of our sacrifice

prevented us from measuring these behaviors. In the current studies, I would have expected to see a dramatic reduction of both food intake and body weight during the dark cycle following the time of sacrifice. Therefore the presented data do not reflect the estrogenic effect on behavior. In hindsight, setting a group of animals aside to be sacrificed immediately following the dark cycle after the plasma peak would have provided a fuller story on how E2 interacts with the diet.

We also found conflicting findings between the exogenous and endogenous E2 studies. While the results in the hypothalamus seemed to match up, we were unable to reproduce the results found in intact females after E2 replacement in the liver. A possible explanation for this is an increased post-surgical inflammatory state. All OVX animals entered the experiment paradigm approximately nine days after surgery. We suggest they might not have been fully recovered and thus may explain for the conflicting results in the liver. In future studies using OVX rats we will plan additional time for recovery prior to the start of an experiment to examine inflammation.

Future Studies

Study 1: **Further investigate the sex differences observed in XBP1 expression in the liver.** We determined that males had increased expression of XBP1 in the liver compared to females. In addition to managing ER stress, XBP1 plays a role in *de novo* lipogenesis (Lee et al., 2008; Glimcher and Lee, 2009). Males generally have increased insulin resistance in the liver compared to females, which would result in increased blood glucose levels (Born et al., 2003). Increases in blood glucose activate the expression of XBP1 to increase *de novo* lipogenesis (Lee et al., 2008; Glimcher and Lee, 2009). This

mechanism may provide a potential explanation to the increased XBP1 observed, but needs to be further assessed. Additionally, we observed that the reduction of XBP1 expression in the liver is E2 dependent; therefore we need to understand better the role of E2 in this phenomenon.

The next step would be to measure triglycerides and FFAs in the plasma, fat content of the liver and the fatty acid synthesis pathway in males and females. Additionally, we would measure protein levels and not just mRNA in the liver to get a better idea of how the liver reacts to a HF diet in males and females. We would also give the HF diet for a longer period of time – possibly for a month so that these differences would be easier to capture.

Study 2: **Investigate the mechanism of gene-specific expression differences in the hypothalamus of HF diet females.** In both experiment 1 and experiment 2, intact females and OVX + E2 females displayed a range of differences in inflammatory gene expression within the hypothalamus. We selected three pro-inflammatory cytokines that are NFκB responsive to determine the effects of E2 on expression. While each cytokine has its own specific role in intracellular signaling, one would expect a similar pattern of expression dependent on the activation of NFκB. E2 affects NFκB response element binding by managing the translocation of co-activators and co-repressors to genes (Ghisletti et al., 2005; Straub, 2007). These data taken together, indicate that the effect of E2 on the NFκB promoter binding may be gene dependent. This may explain the differences observed in hypothalamic gene expression amongst females undergoing diet-induced stress.

To investigate E2-driven changes in the hypothalamus we would use OVX rats and give E2 centrally, again in the 4 day cycle to further investigate the central effects of E2 and to find out if central E2 has the same effects as peripheral E2. We would measure NFκB in the cytoplasm and the nucleus, and measure NFκB responsive genes. To get a better idea of the effects of these genes we would again measure mRNA, and add western blots to see how this is reflected in protein levels.

Study 3: **Determine if females develop central insulin and leptin resistance after 72 hours on a HF diet.** Results from other studies strongly support that 72 hours of a HF diet results in both insulin and leptin resistance in the hypothalamus of male rats (Wang et al., 2001; Morgan et al., 2004). The effects of this time exposure of HF diet on the signaling of these two anorexogenic hormones are not known in females. The reduction in inflammatory signaling, prevention of diet-induced increases in SOCS3, and maintenance of IL-6 expression observed in females may suggest a sex difference would be observed.

We would measure insulin and leptin sensitivity in female rats with intra-third ventricular injections of each hormone. Central leptin and insulin should decrease food intake and body weight. We would compare this on LF and HF diets after 72 hours of a HF diet. Additionally we would measure protein levels of SOCS3 and phosphorylated and unphosphorylated JNK and IRS-1 levels.

Final Statements

Female rodents do not always become obese when fed a HF diet. Due to E2, females are able to adapt to the increased FFAs and maintain central sensitivity to

anorexogenic hormones during diet-induced stress. The findings of my studies suggest that females may be protected during the initiation to a HF diet. This was determined by reduced inflammatory gene expression (TNFα), prevention of HF diet-induced increases in SOCS3 expression, and maintenance of IL-6 expression in the hypothalamus. Additionally, we determined that intact females were protected from increased adiposity during 72 hours of a HF diet.

Additional work will help to put our findings in context. By understanding the role of E2 in protecting rats from the effects of a HF diet in the hypothalamus and liver, investigators may begin to understand why postmenopausal women are at particular increased risk for obesity. In addition, the knowledge gained will contribute to a broader understanding of the role of E2 in inflammation and energy homeostasis.

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APPENDIX

HomeCage Scan

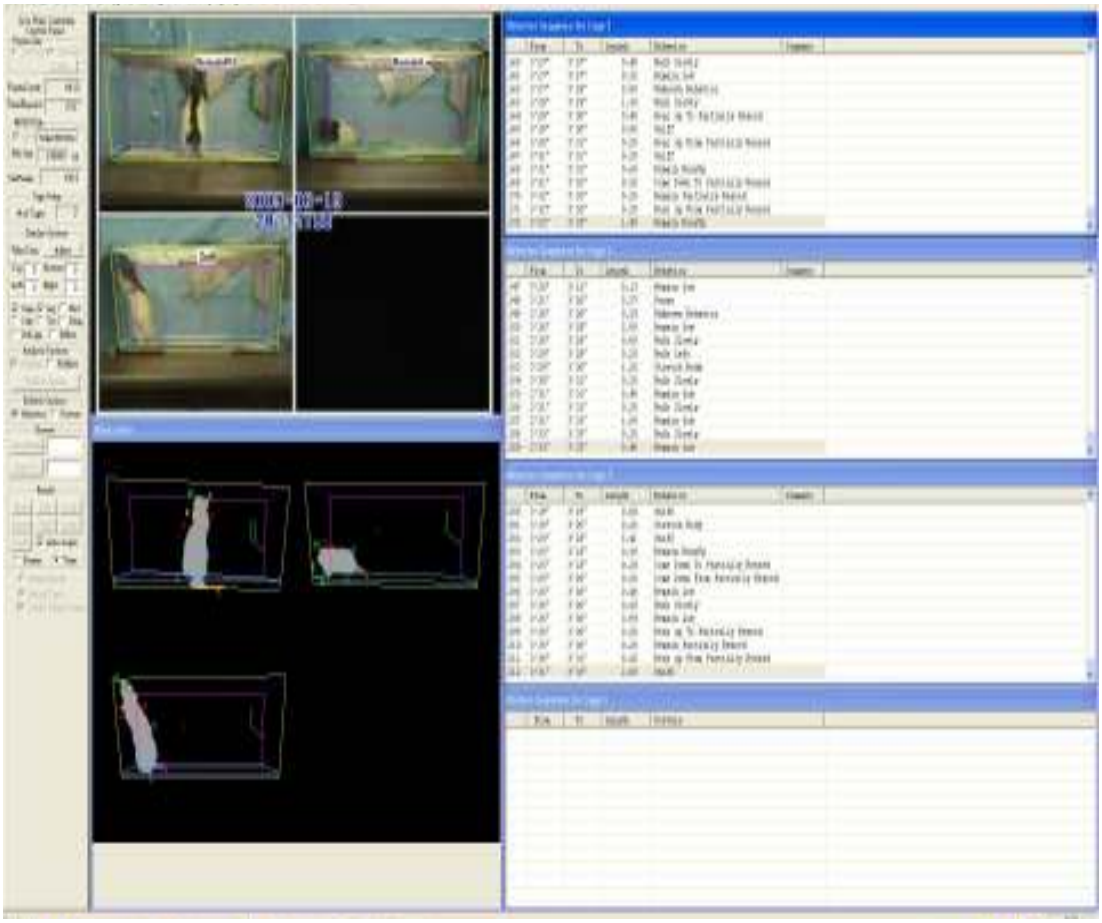

Figure 6-1. **HomeCage Scan-** HomeCage Scan (Clever Systems, Inc) measuring cage activity for each cage. Real-time behaviors are quantified by determining anatomical features of each rat and the location of food and water source to identify behavior.

Specific Methods Developed for the HomeCage Scan

Room Setup

To allow for the discrimination between the white wall and the black and white hooded rats, a blue background was taped on the wall. In addition, blue place cards were kept in between each cage to remove any disturbance in measurement from the adjacent cages and to remove any potential variances in data caused by social interaction.

The room was set at a 12:12 hour light:dark cycle with lights off at 4 pm. To allow the video recorder to see the rats throughout the dark cycle, red lights were shown from underneath each cage onto the blue backdrop. Standard floor lamps with red lights were used throughout the room to provide additional lighting. The lamps providing red light for the dark cycle were kept on at all times. This meant that when the automated timer turned off the white ceiling lights at the end of the light cycle, the red lights allowed the video cameras to view the rats during the dark cycle. Additionally, no one needed to enter the rooms each day to turn on the red lights.

Cage Setup

Food hoppers hung from right back corner of each cage directly behind the water bottle. This placement prevented the hopper from obstructing the view of the rat while allowing for continued and unhindered contact to both food and water. Any other location in the cage would have caused physical obstacles to the rat and accurate measurement of its behavior.

The least amount of cage fill was used during the experiment. While users are able to set the height of the cage fill for each individual cage during the set up of the

program, too much cage fill causes inaccuracies in measurements. In addition to becoming a potential disturbance and providing "noise" around the rat, the levels of the cage fill change throughout the day. Rats will often push around the cage fill, which can result in a change of the original level of fill that was set at the beginning of the experiment. A major change in the environment will render the results obtained inaccurate. Due to these reasons, a light covering approximately a quarter of an inch was distributed evenly in clean cages. This required the cages to be changed on a daily basis throughout the experiment.

Program Setup

The HomeCage Scan program was designed to "see" animals that are either dark or white in coloring. Since Long-Evans rats are hooded and are black and white, we were not able to use the programmed settings and our experiments required individual settings to be created.

Table 6-1. **Program Settings**

	Contact Threshold	Groom Motion Low Threshold
Day	30-33	1.25-1.50
Night	No Change	N/A

Legend: Specific changes to standardized settings to allow for accurate measurement of the Long-Evans rat for use with the HomeCage Scan. Increasing the contact threshold reduces the sensitivity of the measurements causing slightly less accurate results.

Computer Setup

At least 32 gigabytes of free space on a hard drive are needed to record a 24 hour experiment. The hard drives on the computers that are equipped with the HomeCage Scan do not have enough hard drive space to accommodate 72 hours of data. We used external hard drives to alleviate the load placed on the computers. Additionally, video files were deleted daily to provide enough hard drive space for the program to function properly. If this does wasn't done, the program would crash during the experiment and all data would be lost.

Other Housekeeping Tips that Enhance Program Performance

Once the experiment is set up and ready to analyze for extended period of time several additional steps were needed ensure a seamless recording. Both behavior and posture items in the Refresh Options window on the left hand panel should be unchecked. Additionally, any other display options that may be active can be turned off. Outside of the program, any additional software should not be running at the same time and the computer should not be attached to the internet to prevent automatic updates.