

INVESTIGATION INTO THE EFFECTS OF ENALAPRIL ON FOOD INTAKE AND
BODY COMPOSITION IN HIGH-FAT FED AGED RODENTS

By

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To my family and friends, whose humor and encouragement got me through many long nights

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The widespread consumption of a high-fat “Western diet” in conjunction with decreased physical activity has created an obesity epidemic with severe health ramifications in aged individuals. Body composition and obesity may represent important mediators in the age-related onset of physical disability. Our previous studies indicate that, with high-fat feeding, aged rats experience a heightened hyperphagia and a delayed normalization in caloric intake, resulting in an exaggerated weight gain, relative to similarly treated young rats. Angiotensin converting enzyme (ACE) inhibitors attenuate declining physical function in aged rats via modification of body composition and modulation of metabolic and inflammatory pathways. Therefore, we sought to determine if the ACE inhibitor enalapril mitigates this hyperphagic response. Twenty-four month old male, F344x BN rats were administered placebo or enalapril (40 mg/kg/day; in food pellet) for four weeks. Thereafter, half of the placebo rats continued to receive regular chow. The other half of the placebo group and the entire enalapril group were given a high-fat diet for thirteen days. Compared with the high-fat-fed placebo rats, which reached a peak caloric intake on Day 1, enalapril high-fat-fed rats demonstrated a more gradual hyperphagia, peaking at Day 4. Overall, body weight in

the enalapril high-fat-fed remained lower than placebo high-fat-fed, which was mainly attributable to an attenuation of fat mass gain. Thus, in the context of exposure to a high-fat diet, enalapril may impede the development of obesity via direct modification of body weight/composition and an initial reduction in caloric intake.

CHAPTER 1 INTRODUCTION

Overview

Developing preclinical models of late-life intervention strategies for combating declining physical function has enormous significance (de Grey, 2007; Rae et al., 2010). With the continued “graying” of the world-wide population, the number of individuals at risk of developing physical disability continues to increase and the sky-rocketing social, emotional and economic cost (Olshansky et al., 2009) of caring for such individuals mandates the need for testing the effectiveness of health-promoting interventions within this cohort. To address this need, we have used the Fischer 344 x Brown Norway (F344BN) rat as our model, since several studies have shown that this strain proceeds from 80% to 50% mortality between 24 and 30 months of age. In humans, this same pattern of survival mirrors an exponential increase in disability. In fact, in both rats and humans, assessment of functional limitations in the 50% survival range is highly predictive of future disability and ultimately mortality (Carter et al., 2002; Guralnik et al., 1994). As a result of rising economic costs and reduced overall well-being of our aging populations, it is critical to determine the biological processes and lifestyle factors that contribute to decline in function. Reduced physical function is concurrent with aging, and often limits the ability to perform activities of daily living, or ADLS, which includes basic self-care such as bathing, dressing, and feeding. The inability to perform these basic functions has a significant impact on the physical and psychological well-being of elderly individuals as it often becomes necessary for these individuals to enter nursing-care facilities, which limits independence and increases economic burden. In addition, a number of age-related diseases such as cardiovascular disease and type II diabetes

lead to increased morbidity, mortality (Masoro & Austad, 2006, p.45), and contribute to ADL disability.

Changes in body composition are a natural part of the aging process. A principal biological process called sarcopenia, or skeletal muscle atrophy, has been identified as a principal contributor to physical disability. Sarcopenia, which is derived from Greek meaning “poverty of flesh” (Rosenberg, 1997), is associated with age-related changes in body composition and is highly correlated with reduced physical performance (Buford et al., 2010). Sarcopenia and the co-occurrence of obesity, however, involves the invasion of adipocytes into skeletal muscle tissue and precipitates a multitude of diseases, including inflammation, insulin resistance, and cardiovascular disease. The Western lifestyle is infamous for the consumption of a highly processed, fatty diet and lack of exercise. Metabolism slows and adiposity increases as we age, therefore elderly individuals may be more susceptible to the adverse health effects of a high-fat diet. Therefore, one of our future aims is to determine the effect of a high fat diet on the progression of sarcopenia and whether pharmacological interventions, such as angiotensin converting enzyme inhibitors (ACE inhibitors), may mitigate the effects of a high fat diet.

ACE inhibitors have been shown to be highly effective in mitigating body weight and fat tissue gain, improving insulin signaling, and reducing levels of inflammatory cytokines (Santos et al., 2009; Carter et al., 2004). Physical function studies in rodents have revealed that enalapril treatment in older animals attenuates both age-related increases in adiposity and decline in physical function (Carter et al., 2004). Thus, our present study was designed to establish preliminary data regarding the effects of ACE

inhibitors in the presence of a high fat diet. Although exercise is a primary defense against obesity, it may not be recommended for older individuals who may present with conditions such as congestive heart failure (CHF), arthritis, or obesity that precludes them from exercising comfortably. Therefore, development of pharmacological interventions would be ideal for this population, and the use of enalapril as an adjuvant may increase the ability of these individuals to engage in exercise. ACE inhibitors may potentially be used as effective alternatives and provide the health benefits of diet and exercise. We hypothesize that the use of ACE inhibitors will mitigate the adverse effects of a high-fat diet, and our current study may allow for future investigations focused on determining whether ACE inhibition drugs will maintain physical function in the presence of a high fat diet.

Rodent Model for Declining Physical Function with Age

In aged humans, sarcopenia and concomitant adiposity result in reduced strength and physical function (Newman et al., 2003; Goodpaster et al., 2001). In order to test hypotheses regarding effective drug and lifestyle interventions, researchers need an animal model that undergoes similar changes in body composition and physical function. Thus, we use the Fischer 344 x Brown Norway (F344 x BN) rat for aging studies because the age-related changes in body composition, strength, and longevity mimic those in human subjects (Turturro et al., 1999; Carter et al., 2004; Rice et al., 2005). F344 x BN rats, which have an average lifespan of 30 months, gain fat and lean mass from 3 to 24 months of age, lose lean mass and continue to gain fat mass from 24 to 27 months, followed by a decline in both tissue types from 24 to 30 months (Rice et al., 2005; Carter et al., 2004). The changes in body composition reflect the underlying changes in the cell-signaling pathways in adipose and muscle tissue, and resultant

pathophysiologies, such as insulin resistance and inflammation, are also observed in humans (Chung et al., 2009). The physiological changes that occur in F344 x BN rats are similar to those in humans, and can be used as a predictor for physical decline and mortality (Carter et al., 2010). Since the F344x BN model closely mimics human lifespan and age-related changes in body composition, we used this rodent model to assess the potential of late-life pharmacological interventions in mitigating adverse changes in body composition, such as increased adiposity, in the presence of a high fat diet.

Sarcopenia and Age-Related Increases in Adiposity

Sarcopenia, a natural part of the aging process, may be the principle causative factor for physical decline with age. However, a definitive criteria for the condition has not been established; present definitions are derived from epidemiological studies that base criteria on measures of upper and lower body strength and/or characterized levels of muscle mass (Baumgartner, 2000; Cruz-Jentoft et al., 2010; Visser, 2009). In order to demonstrate a correlation between reduced physical function and sarcopenia, changes in both strength and muscle type must be considered. Manini and Clark coined the term dynapenia, derived from the Greek “poverty of strength,” which refers to the loss of strength that occurs with age (Clark & Manini, 2008). Prior studies show that reduced muscle mass is not proportional to loss of strength, and that changes in muscle quality with age lead to reduced strength (Carter et al., 2005). In addition, treatments with growth hormone or testosterone supplementation, which cause muscle hypertrophy, do not result in proportionate increases in strength (Clark & Manini, 2008; Clark & Manini, 2010). Thus, it is clear that age-related changes in biological pathways affecting or occurring within muscle tissue are the primary causes of diminished strength.

Reduction in muscle quality is associated with increased adiposity that is independent of weight gain (Kershaw & Flier, 2004) and exacerbated by the invasion of adipose cells into muscle tissues (Buford et al., 2010). Humans experience a continuous increase in adiposity and reduction in lean tissue from 25 to 65 years of age, after which overall body weight declines (Schwartz, 1998). Lipotoxicity resulting from invasion of adipose tissue into skeletal muscle can lead to the dysregulation of muscle function and increased apoptosis (Kusminski et al., 2009). In addition, the increase in fat tissue leads to multiple pathologies, including chronic inflammation, insulin resistance, and oxidative stress, all of which exacerbate reduction in the quantity and quality of skeletal muscle (Jensen & Hsiao, 2010). Adipose tissue secretes a multitude of inflammatory cytokines, such as IL-6 and tumor necrosis factor- α (TNF α), and immune-related proteins. TNF α suppresses expression of genes involved in uptake and metabolism of glucose, such as IRS-1 and GLUT4, and suppresses gene activity involved in the breakdown of fatty acids. In addition, TNF α enhances expression of genes that promote synthesis of fatty acids and cholesterol. IL-6 is also highly correlated with insulin resistance and obesity, and is used as a predictor for the development of type II diabetes and cardiovascular disease (Kershaw & Flier, 2004). Increased adiposity subsequently results in over-expression of cytokines, promoting insulin resistance, whole-body inflammation, and reduced lifespan. Establishing an effective pharmaceutical intervention, such as ACE inhibitor treatment, that successfully mediates weight and adipose gain associated with a high fat diet, may allow for future investigation that determines if these interventions subsequently reduce inflammation and improve insulin sensitivity in aged animals.

The Impact of Sarcopenia and Concomitant Obesity on Physical Function

In humans, muscle mass and strength begins to deteriorate at around age 30, with an accelerated loss occurring after age 60 (Goodpaster et al., 2001; Stenholm et al., 2008). Visceral and intramuscular adipose tissues proliferate while subcutaneous fat is diminished (Stenholm et al., 2008). A progressive increase in fat mass is a normal part of the aging process, even in the absence of considerable changes in body mass index (Zamboni et al., 2008). The reduced basal metabolic rate (BMR) observed in elderly individuals has been attributed to loss of muscle mass, while decreased physical activity aggravates muscle atrophy and weight gain (Stenholm et al., 2008; Evans & Campbell, 1993). Thus, it appears that reduced physical activity accelerates BMR reduction and subsequently intensifies weight and fat tissue gain. Age-related obesity is regarded as a significant risk factor for a myriad of chronic diseases, such as metabolic dysregulation (i.e. insulin and leptin resistance), increased inflammation, and dyslipidemia (Kershaw & Flier, 2004; Evans & Campbell, 1993). The conventional assumption that adipose tissue is an inert receptacle for energy storage is false; adipose tissue secretes a variety of hormones, interacts with the central nervous system, and responds to hormone systems. Thus, excessive or insufficient fat reserves can have adverse effects on metabolic function (Kershaw & Flier, 2004). The chronic inflammation resulting from increased adiposity may also contribute to muscle mass degeneration and accelerate age-related muscle loss (Jensen & Hsiao, 2010).

As of 2010, 30% of adults between ages 60 and 79 were obese, with a BMI ≥ 30 kg/m². Obesity is becoming more prevalent among older individuals, and is associated with reduced physical function and chronic disease (Jensen & Hsiao, 2010). Sarcopenia in conjunction with obesity is associated with adipocyte infiltration into lean muscle

tissue, or myosteatorsis (Thornell, 2011). The intrusion of adipocytes into muscle tissue has been correlated with reduced lower body strength and disability. Muscle strength is measured in terms of force and power; power is proportional to muscle volume, whereas force is proportional on the cross-sectional area (CSA) of the muscle. Muscle power, which is responsible for everyday activities such as rising from a chair or climbing stairs, declines to a greater degree than force in aged individuals and therefore contributes to disability and loss of independence. Obese aged individuals are at a much greater risk of disability; not only do they have age-related reduction in CSA, but they also need extra power to overcome the weight of excess adipose tissue (Narici & Maffulli, 2010; Stenholm et al., 2008). In addition, obesity is strongly correlated with self-reported reduction in physical function; the probability of an elderly individual experiencing reduced physical function doubles in the presence of moderate obesity, and quadruples with severe obesity (Jensen, 2005). Recent studies have indicated that a positive correlation exists between waist circumference, an indication of visceral adiposity, and increased risk of functional decline (Jensen & Hsiao, 2010). Thus, aged obese individuals are at a much higher risk of experiencing rapid physical decline, frailty, and disability. The development of pharmacological interventions, such as ACE inhibitor treatment, may mitigate obesity in the presence of a high-fat diet, possibly preventing the development of metabolic dysregulation and inflammatory processes.

Modulation of Body Composition and Gene Expression by Angiotensin Converting Enzyme Inhibitors

The renin-angiotensin system (RAS) is the principal regulator of blood pressure, and involves a cascade of peptide transformations. Angiotensinogen, an inactive globulin produced by the liver, is cleaved by renin to form angiotensin I (Ang I), which is

subsequently converted to angiotensin II (Ang II) by angiotensin-converting enzyme, or ACE. In addition, ACE induces proteolysis of bradykinin. Ang II is a potent vasoconstrictor while BK is a potent vasodilator, so the formation of Ang II and degradation of bradykinin by ACE results in increased blood pressure. Thus, ACE inhibitors serve to lower blood pressure by blocking the conversion of Ang I to Ang II and arresting degradation of bradykinin (Carter et al., 2005). ACE inhibitors not only lower blood pressure, but also improve physical function via direct modulation of body composition.

The Health, Aging and Body Composition Study (Health ABC Study) compared the size of the lower extremity muscle mass, or LEMM, in a cohort of elderly individuals aged 70-79 who were using various antihypertensive drugs, including ACE inhibitors. The data revealed that individuals taking ACE inhibitors had a significantly larger LEMM than those taking other antihypertensive medications, indicating a direct effect of ACE inhibitors on body composition (Onder et al., 2002). Daily injections of the ACE inhibitor enalapril (40 or 80 mg/kg/day) over the course of six months reduced adiposity and improved physical performance in aged F344xBN rodents (Carter et al., 2004). Furthermore, upregulation of Ang II and angiotensinogen expression in rodents resulted in proliferation of adipocytes and increased triglycerides (Engeli et al., 2000). The data validate that the peptides involved in the RAS cascade directly modify body composition, and therefore, ACE inhibitors can mitigate these effects.

ACE inhibitors have been shown to attenuate age-related physical decline and mitigate adipose tissue gain in both humans and rats in the absence of antihypertensive effects (Onder et al., 2002). A three-year evaluation of walking speed and knee

extensor muscle strength in hypertensive women over the age of 65 with moderate to severe disability was conducted to evaluate the effects of ACE inhibitors on functional decline. Of the 641 subjects, 61 had continuously used ACE inhibitors, 301 used alternate antihypertensive medications either continuously or intermittently, 133 intermittently, and 146 never. Researchers found that subjects who used ACE inhibitors continuously had a lower average decline in muscle strength and walking speed (Onder et al., 2002). After adjusting for the occurrence of congestive heart failure, myocardial infarction, and stroke, the results remained unchanged, indicating that ACE inhibitors act independently of cardiovascular events (Onder et al., 2002).

Apoptosis (i.e., programmed cell death) may play a key role in the development of sarcopenia. In particular, the apoptotic pathway triggered by TNF- α is thought to play a central role in the pathogenesis of age-related muscle loss (Chen & Goeddel, 2002). Age-related changes in the secretion of TNF- α from adipose tissue assessed in F344x BN rats indicate that TNF- α protein expression/ mg of total protein increases substantially between 20 and 30 months of age and that this increase occurs primarily in intra-abdominal fat. We used Q-PCR to evaluate TNF- α gene expression in the tibialis anterior (TA) muscle of aged rodents treated with either enalapril or N(G)-nitro-L-arginine methyl ester (L-NAME), a nitric oxide blocker. We found that TNF- α levels were reduced with enalapril treatment, an effect that was reversed in L-NAME treated rodents. In addition, we performed gene array analyses in order to determine which cell signaling pathways are altered by enalapril. RNA was isolated from the soleus muscle of rats treated with either placebo or 40 mg/kg/day of enalapril. We found that 237 transcripts were significantly up-regulated and 77 were significantly down-regulated by

enalapril (Carter et al., 2010). Using IPA, we generated and scored 25 functional networks based on transcripts differentially expressed between groups. The IPA network with the highest score consisted primarily of genes related to cellular maintenance, including various genes related to apoptosis regulation. In fact, p53 and the caspases were found to be at the heart of this network, indicating that enalapril's effects on aged skeletal muscle may be mainly attributed to regulation of apoptosis (Carter et al., 2010). Using western blot analysis, we next evaluated enalapril's effects on apoptosis in the gastrocnemius muscle of rats given either 20 or 40 mg/kg enalapril, 30 mg/kg losartan, or placebo between 24 and 27 months of age. We found that enalapril-treated rats had significantly decreased levels of DNA fragmentation compared to their losartan and placebo treated counterparts. In addition, we observed a down-regulation of cytochrome c, active caspase-9, and cleaved caspase-3, all markers specific to the mitochondrial caspase-dependent apoptotic pathway (Carter et al., 2010). These data show that enalapril may exert its beneficial effects on aged skeletal muscle by influencing gene expression.

ACE inhibitors also have the ability to alleviate the metabolic dysregulation and inflammation associated with obesity. For example, ACE inhibitors may improve insulin sensitivity by increasing circulating levels of bradykinin. Bradykinin binds to the bradykinin B2 receptors on adipocytes, increasing tyrosine phosphorylation of insulin receptors on the adipocytes (Engeli et al., 2000). In turn, intracellular substrates, such as insulin-receptor substrate-1 (IRS-1) are activated and downstream insulin signaling-pathways are initiated, which may enhance insulin signaling and glucose uptake (Olivares-Reyes et al., 2009).

Previous studies in our lab show that enalapril treatment can mitigate age-related gains in adiposity and functional decline. Unpublished data from our lab established that enalapril doses less than 20 mg/kg are ineffective (Carter et al., 2004), and therefore studies typically use at least 20 to 40 mg/kg dosages. A prior study assessed the effects of enalapril on physical performance and body composition; male F344 x BN rats were injected daily with either enalapril (40 mg/kg or 80 mg/kg) or saline from 24 to 30 months of age. Physical performance was evaluated via grip strength and inclined plane tests. In humans, grip strength is a predictive tool for disability, and stamina and muscle tone are indicative of muscle quality (Rantanen et al., 1999). Forelimb grip strength was assessed with an automated grip strength meter. The mean force was calculated and divided by the animal's body weight. The inclined plane test involved placing animals in an upward position on a 60° inclined screen. The time taken for the animal to fall onto the foam pads below were divided by the animal's weight. The physical performance data was evaluated based on data from animals that survived for the full six month treatment period, as it was hypothesized that changes in physical performance may not be apparent until the end of the study. There was no difference between the two enalapril groups; however, comparison of the combined enalapril data to the saline group revealed that the saline-treated animals had a significantly greater decline in inclined plane performance, and a moderately significant decline in grip strength. As for body composition, both enalapril groups lost significant proportions of adipose tissue between 27 and 30 months, while the saline group gained fat tissue. Loss of lean mass was equivalent between the enalapril and saline groups between 24 and 27 months of age, with lean mass stabilizing thereafter (Carter et al., 2004). These results

demonstrate that enalapril is effective in attenuating functional decline. The significant reduction in overall adiposity in enalapril-treated animals may account for this phenomenon, since there was no difference in lean mass, muscle area, or fiber loss between the enalapril and saline groups.

A six-month longitudinal study compared the effects of enalapril and losartan, an angiotensin receptor blocker (ARB), on physical function, body weight and body composition on aged rats. ARB and ACE inhibitor treatments were evaluated to discern if the two drugs exert similar physiological effects, such as equivalent changes in body composition. In addition, comparison of the drugs' effects may help elucidate the biological mechanisms of ACE inhibitors and their contribution to reduced physical performance and changing body composition. The ACE inhibitor enalapril modulates the RAS by blocking the conversion of Ang I to Ang II. However, the ARB losartan antagonizes AT1, the Ang II receptor, and prevents Ang II physiological action without obstructing other ACE pathways. 24 month old rats were given 40 mg/kg enalapril, 30 mg/kg losartan, or placebo. Body composition and physical performance were assessed at baseline (24 months old), and at 27 and 30 months of age. Food intake, body temperature, glucose and insulin levels, and tumor development were also evaluated in order to determine if outcomes were directly related to treatment or to separate mechanisms. At 27 months, differences in physical function, body weight, and body composition were significant, although by the conclusion of the study, these differences were negligible. Enalapril was shown to be effective in reducing adiposity and body weight, with a maximal effect occurring within the first three months of the study. Throughout the study, enalapril-treated rats had a lower overall average body weight

than losartan or placebo-treated rats. Animals in the losartan and placebo groups, however, experienced an increase in adiposity between 24 and 27 months of age. Physical performance was evaluated by measuring grip strength. Enalapril and losartan were both effective in mitigating age-related decline in grip strength. In contrast to enalapril, losartan attenuated functional decline in the absence of changes in body weight or composition, indicating that both drugs exert their effects by targeting skeletal muscle. In order to test this hypothesis, gene array analysis was used to determine which pathways are acted upon by enalapril. Gene array data indicated that the direct effects of enalapril and losartan may be attributed to apoptosis. The ELISA method was used to determine the extent of apoptosis in gastrocnemius muscle, which is known for undergoing substantial age-related degeneration and elevation in apoptosis markers. Data showed that only enalapril was effective in attenuating apoptotic activity in gastrocnemius muscle. Finally, enalapril-treated animals did not undergo any changes in body temperature, food intake, or physical activity that would account for the observed effects on body composition or physical function. Relative to the losartan and placebo groups, enalapril effectively reduced age-related tumorigenesis. Enalapril-treated animals decreased food consumption between 24 and 27 months of age, even in the presence of increasing adiposity (Carter et al., 2010). These data lends credence to the idea that enalapril may mitigate obesity by alternate pathways, such as increased fatty acid oxidation or improved insulin signaling, instead of through lowered caloric intake or leptin signaling pathways.

Physiological Effects of Long-Term ACE inhibitor Treatment in Young Animals

Studies in 3 month old Wistar rats demonstrated the life-prolonging effects of ACE inhibitor treatment during administration of a high-fat diet (Santos et al., 2009). The

animals were divided into regular chow and hyperlipidic diet groups. Approximately half of the animals in each group served as a control group, with the other half receiving enalapril treatment for one month. Body weight, body composition, leptin transport across the blood brain barrier (BBB), and levels of antioxidants were examined in order to determine if enalapril could reduce the detrimental effects associated with a high calorie diet. The data revealed that the high-fat fed animals treated with enalapril had a prolonged lifespan compared to high-fat fed control animals. The increase in lifespan can likely be attributed to mitigation of weight gain, reduction in inflammatory cytokines, and improved insulin signaling. Suppressed ACE activity in enalapril-treated animals enhanced expression of PPAR γ , which increases metabolism and reduces circulating levels of glucose, insulin, and triglycerides. In addition, adiponectin expression was increased, contributing to decreased glucose levels, inflammation, and upregulation of fatty acid catabolism. Catalase and superoxide dismutase (SOD), which are responsible for minimizing levels of hydrogen peroxide and superoxide radicals, were increased in enalapril-treated animals, promoting longer lifespan. Lastly, enalapril did not promote leptin transport across the BBB, nor did it enhance CNS sensitivity. Circulating leptin levels were lower in enalapril-treated animals, which can be attributed to their lower overall fat mass (Santos et al., 2009). However, the direct physiological effects of enalapril indicate that despite its inability to enhance leptin signaling, enalapril is an effective drug for mitigating the pathologies associated with a hyperlipidic diet and subsequent obesity.

Future Potential for Enalapril Treatment

Enalapril has the potential to prolong quality of life and lifespan in elderly and obese individuals. Enalapril has been highly effective in reducing adiposity and

increasing physical function, outcomes that can be attributed to reduced inflammation, improved insulin signaling, and increased antioxidant levels. With a growing elderly population and a widespread obesity epidemic, researchers hope enalapril can reduce associated disease processes in these individuals. As previously discussed, enalapril has been highly effective in mitigating obesity and pathologies in high-fat fed young animals. However, it is currently unknown if enalapril will produce the same results in aged animals. Thus, the goal of the research is to investigate whether high fat fed aged animals pre-treated with enalapril will experience the same effects. Enalapril effectively reduces adipose tissue and circulating levels of leptin. Thus, it is possible that pretreating aged animals with enalapril will reduce hyperleptinemia and moderate leptin resistance. Given that we will not have the leptin data from this experiment to support this idea, we are using this study to establish a model for future experiments involving enalapril's effect on leptin resistance in aged animals. The present study will be used to evaluate three main hypotheses: pretreatment of high-fat fed aged animals with enalapril will result in (1) reduced hyperphagic response, (2) spontaneous division into the diet-induced obesity (DIO) and diet-resistant (DR) groups observed in younger animals, and (3) modulate the increase in overall adiposity.

CHAPTER 2 EXPERIMENTAL DESIGN AND METHODS

Animals

56 Male Fischer 344 x Brown Norway (F344 x BN) rats were purchased from the National Institutes on Aging (NIA) Colony at Harlan Industries (Indianapolis, IN). Animals were received at 24 months of age and housed individually under standard temperature and lighting conditions (12 hour light:dark cycle) in a specific pathogen-free facility accredited by the American Association for Accreditation of Laboratory Animal Care. Water was provided *ad libitum* at all times. The University of Florida's Animal Care and Use Committee approved all protocols. Health checks were performed daily in order to identify injury or disease.

Diets

Bacon-flavored food pellets were purchased from BioServ, Frenchtown, NJ. 40 mg/kg of enalapril was compounded into the bacon pellets; placebo pellets did not contain drug. High-fat rodent chow was purchased from Research Diets Inc, New Brunswick, NJ and consisted of 60% fat, 20% protein, and 20% carbohydrate. Individual food pellets contained 5.24 kcal/gram of metabolizable energy. Regular Harlan® Teklad rodent chow was obtained from Harlan Laboratories Inc, and was composed of 4% fat, 24% protein, and 4.5% fiber. Individual food pellets contained 3.1 kcal/gram of metabolizable energy.

Experimental Design

Upon arrival, animals were acclimated to the new environment (i.e. no handling by lab personnel) for one week, followed by a five day acclimation to handling. Immediately following handling acclimation, and at the start of weekly food and body

weights, the animals were randomly divided into a placebo (n=31) and enalapril (n=25) group; animals were randomized so that the average weight of the two groups were equivalent. Drug acclimation began four days after dividing the animals into the placebo and enalapril groups. Three weeks after the start of the drug acclimation period, the placebo group was divided into a placebo high fat (n=17) and placebo regular chow (n=14) group. All enalapril animals were assigned to receive high fat chow. Animals were divided so that the average weight of each group was equivalent. Approximately 5 weeks after the start of the drug acclimation phase, baseline TDNMR was performed in order to record baseline body composition and weight. The high fat diet began three days after TDNMR. Thereafter, food intake and body weights were recorded daily in order to capture hyperphagia and subsequent weight gain. One day after the end of the high fat diet, a second TDNMR was performed in order to record changes in body composition and weight as a result of the high fat chow diet (Figure 2-1).

Food Intake

Recording of food consumption (in grams and kilocalories) began four days before the start of the drug treatment phase and continued throughout the study. Food intake was calculated by recording the quantity of chow remaining and subtracting it from the previous week's (or day's) values. The difference was multiplied by the number of metabolizable kcal/gram (Harlan® Teklad Rodent Diet #8604 (Regular Chow): 3.1kcal/gram. Research Diets #D12492 (High Fat Diet): 5.24 kcal/g). Food intake was recorded on a weekly basis during the drug treatment phase in order to establish baseline consumption levels. Daily recording of food intake began at the start of high fat diet and continued for the duration of the study. Daily observation of intake allowed us to observe the initial hyperphagic response to the high fat diet and determine if the

enalapril and placebo groups responded differently to the highly palatable, energy-dense chow.

Body Weights

Body weights were recorded (in grams) by placing the rodents in an open tupperware container on a digital scale. Values were rounded to the nearest whole number. Baseline body weights were recorded four days before the start of the drug treatment phase, and recorded on a weekly basis thereafter in order to determine the drug's effect on body weight. Upon initiation of the high fat diet, body weights were recorded on a daily basis in order to observe the effects of the hyperphagic response on body weight, and determine if enalapril was effective in mitigating weight gain.

Body Composition

Time-domain nuclear magnetic resonance (TD-NMR) was used to determine changes in body fat and lean mass during each diet phase. TD-NMR was performed at baseline and at the beginning and end of each diet phase. Live, conscious (i.e. no anesthetic) rats were restrained in sample holders (90 mm diameter and ~250 mm length). Screw-cap plungers were adjusted in order to restrain the animals while adjusting for their individual size. The sample holder was inserted into the MiniSpec (TD-NMR Minispec, Bruker Optics, The Woodlands, TX, USA), which analyzed body composition via acquisition and determination of TD-NMR signals from protons in the sample area. Each animal was scanned twice, with each scan averaging two minutes.

Drug Treatment

Animals were randomly assigned to receive 40 mg/kg/day enalapril (n=25) or placebo (n=31) for a period of four weeks. Food and body weights were recorded at the end of each week in order to track changes due to drug treatment.

High Fat Diet (HFD)

After the drug treatment period, the animals were placed on a thirteen day high fat diet. All enalapril animals were placed on HFD, and approximately half of the placebo group (n=17) was placed on high-fat chow, with the other half continuing on regular chow (n=14). Food intake and body weights were recorded daily.

Statistical Analyses

Statistical analyses were performed using SAS® 9.1 for Windows (Cary, NC). Where appropriate, we used multivariate regression models when involving time in the analyses. If the data did not meet the assumptions of the ANOVA, we transformed them using log or square root transformation. We tested for the main effects of treatment condition (placebo high fat; placebo chow; enalapril high fat), time (days) and their interaction. Multiple pairwise group comparisons were performed using the Bonferroni procedure. The level of significance was set at $p < 0.05$ for all analyses. All data are presented as means \pm standard error.

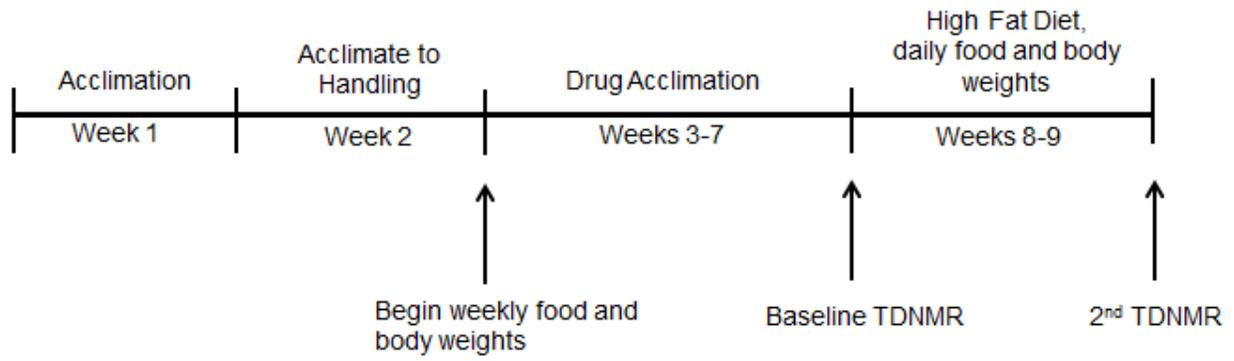


Figure 2-1. Experimental timeline.

CHAPTER 3 RESULTS

Food Intake

In order to determine if the enalapril HF group experienced spontaneous division into diet induced obesity (DIO) and diet-resistant (DR) groups, the total kcals consumed per animal during the first three days of the high-fat diet were evaluated for each group. Figure 3-1 demonstrates that the three groups are distinctly different in terms of kcal intake during the first three days of the diet. Although there is a small overlap in consumption between the enalapril HF and placebo HF groups, the enalapril HF group's intake lies primarily between that of the placebo HF and placebo chow groups. Thus, the enalapril HF group did not divide into DIO and DR groups, but there are distinct differences in intake among the three groups.

Consumption was measured in both kcals and absolute gram intake over the entire 13 days of high-fat feeding (Figure 3-2A and B). On average, there was no difference in either measure ($p_s = 0.483$ and 0.500 respectively). In contrast, during the hyperphagic phase of the experiment (Figure 3-3 A and B; baseline to day 0, 1, 2) there was a main effect of treatment condition ($p < 0.001$), time ($p < 0.001$) and a condition x time interaction ($p < 0.001$) for both kcal and gram intake. Post-hoc analyses demonstrated that across the first three days of high-fat feeding gram intake was no different at baseline amongst all groups. By day 1, the placebo chow and enalapril HF were not different from each other ($p > 0.05$); whereas both groups were different from placebo HF (both $p_s < 0.001$). By day 2 all groups were different from each other, such that placebo HF was greater than enalapril HF which was also greater than placebo chow (all $p_s < 0.001$). The same trend was observed for kcal intake. These data

demonstrate that enalapril attenuates the hyperphagic response observed in aged rats to the presentation of high-fat feeding.

Body Weight

Figure 3-4 shows that during the entire 13 days of the high-fat feeding phase of the experiment, there was a main effect of condition ($p = 0.005$), time ($p < 0.001$) and a condition x time interaction ($p < 0.001$). Post-hoc analyses of the interaction revealed that by day six differences emerged between the placebo HF and placebo chow groups ($p < 0.05$); however no differences were observed between placebo chow and enalapril HF groups nor between the placebo HF and enalapril HF groups (all $ps > 0.05$). By day nine and throughout the remainder of the experiment, all groups were significantly different from each other such that the placebo HF animals demonstrated a larger weight gain than both the enalapril HF and placebo chow groups which were also significantly different from each other (all $ps < 0.05$). Indeed, the overall percent change in body weight from base line to the end of high-fat feeding followed a similar trend (all $ps < 0.05$) (Figure 3-5). These data show that while all high-fat fed animals gained a significant amount of body weight, enalapril mitigated the weight gain predicted with high-fat feeding.

During the hyperphagic phase of the experiment (baseline to day 0, 1 and 2) there was a main effect of treatment ($p = 0.02$), but no effect of time ($p = 0.41$), nor an interaction of the two ($p = 0.96$) on body weight. Overall body weight remained consistent over this time period in both the placebo chow and enalapril HF groups; whereas by day 2, body weight in the placebo HF was significantly higher than the other two groups (both $ps < 0.05$; Figure 3-6). These data demonstrate that body weight over both the hyperphagic and long-term phases of the experiment was significantly

influenced by food intake (both grams and kcals) during the hyperphagic phase of the experiment.

Body Composition

Body composition was assessed at baseline and then after the 13 days of high-fat feeding. There was a main effect of condition ($p < 0.001$, $p = 0.003$), time (both $ps < 0.001$) and a condition x time interaction ($p < 0.001$; 0.009), all respectively for fat and lean mass. At baseline, there were no differences amongst groups for either fat or lean mass grams (all $ps > 0.05$). However, after 13 days of high-fat feeding, all groups were different from each other such that placebo HF animals demonstrated significantly higher fat and lean mass relative to both enalapril HF ($p = 0.05$ and $p = 0.022$) and placebo chow animals ($p < 0.001$ and $p = 0.007$) respectively (Figure 3-7 A and B). In addition, there was no difference between enalapril HF and placebo chow for lean mass ($p = 0.11$) but a significant difference for fat mass ($p < 0.001$). These data demonstrate that enalapril treatment attenuates high-fat feeding induced changes in increases in fat mass.

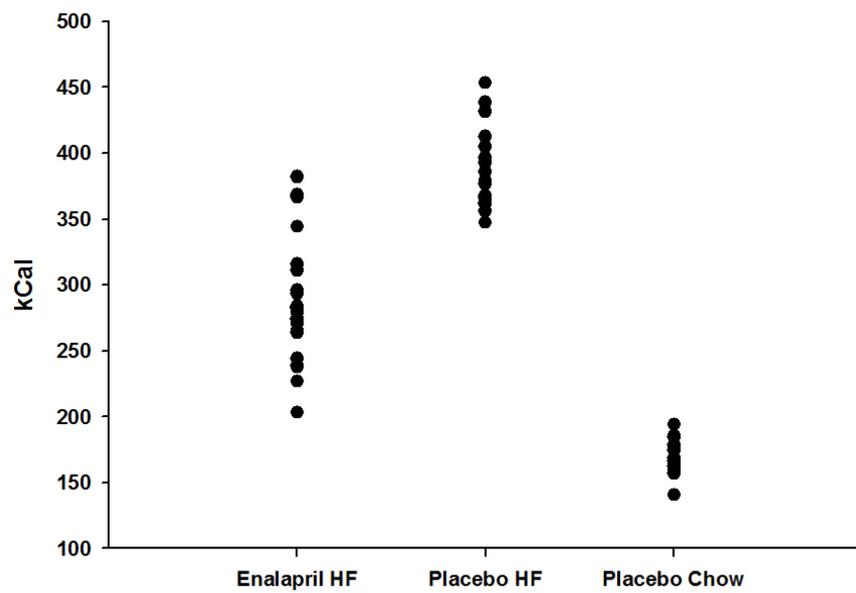
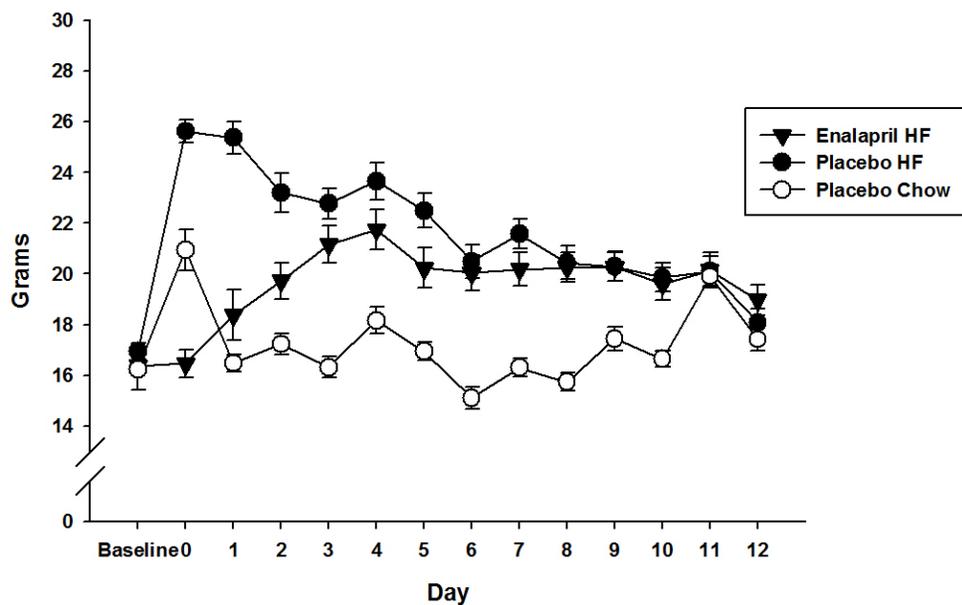
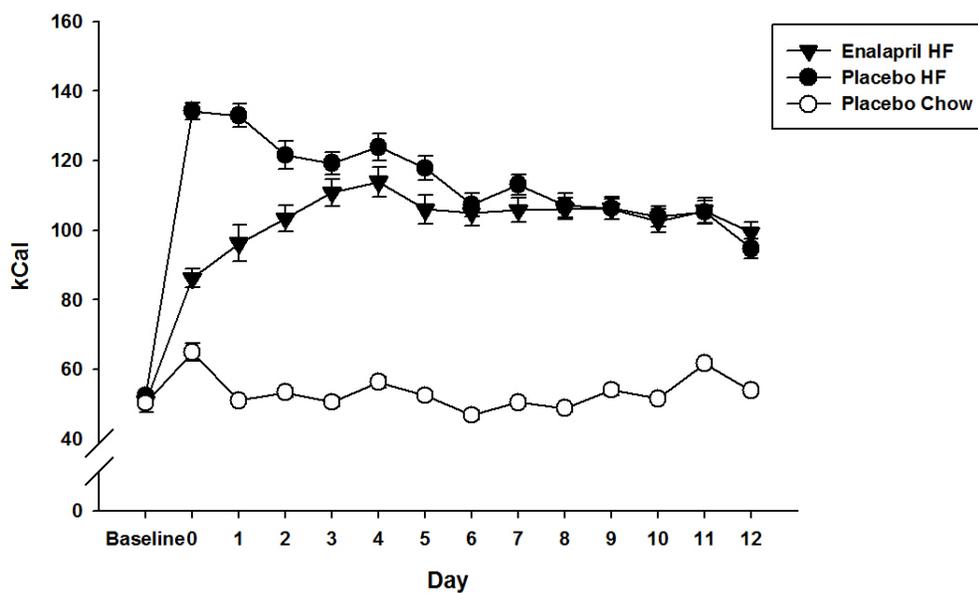


Figure 3-1. Scattergram of total kcal (combined total of days 0,1, and 2) intake per animal per group over the first three days of the high-fat diet.

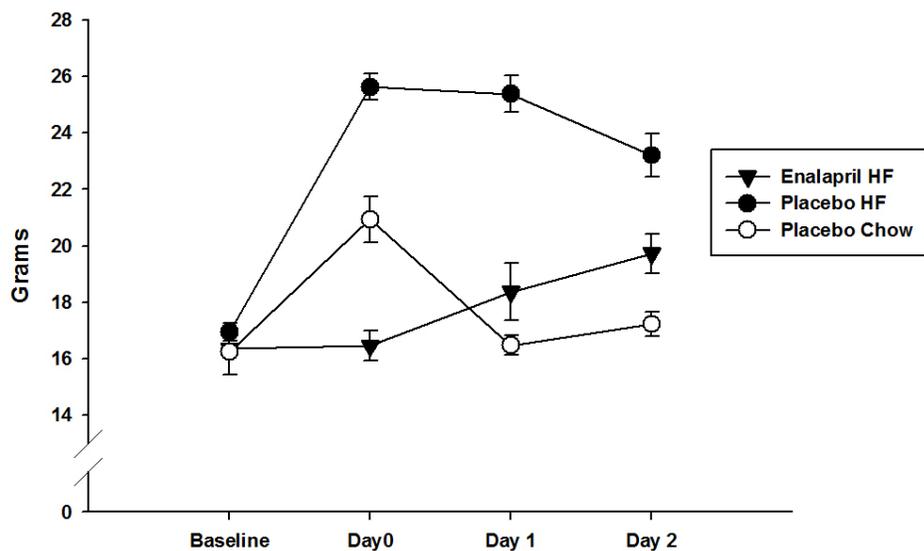


A

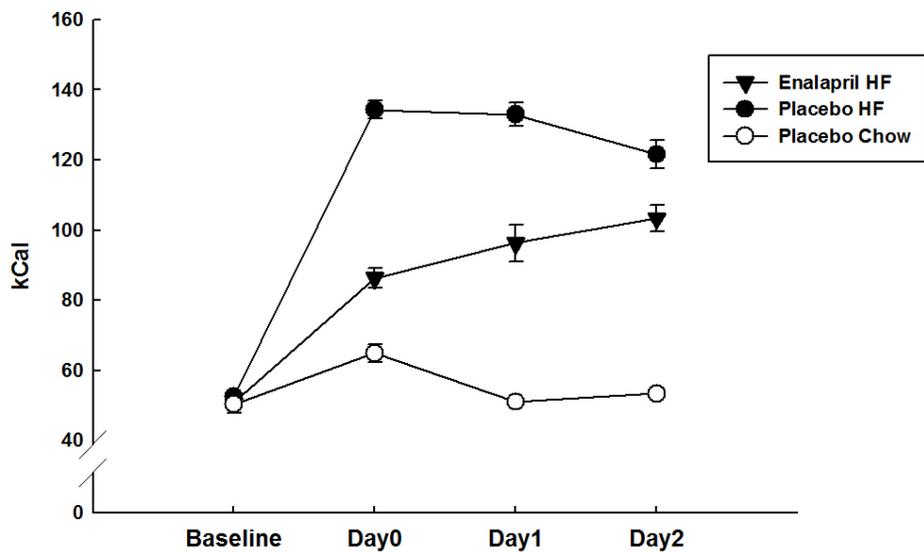


B

Figure 3-2. Food intake over the thirteen-day high fat diet. Days 0 through 12 indicate the first through thirteenth day of the diet. Error bars signify the SEM, or standard error of the mean, for each value. Baseline corresponds to food intake before initiation of the high fat chow diet. A) Food intake in grams. B) Food intake in kcal.



A



B

Figure 3-3. Food intake showing hyperphagia over the first three days of the high-fat chow diet. Baseline intake corresponds to chow consumption before the start of the high fat diet. Days 0, 1, and 2 indicate the first, second, and third days of the diet, respectively. Error bars signify the SEM, or standard error of the mean, for each value. A) Food intake in grams, B) Food intake in kcals.

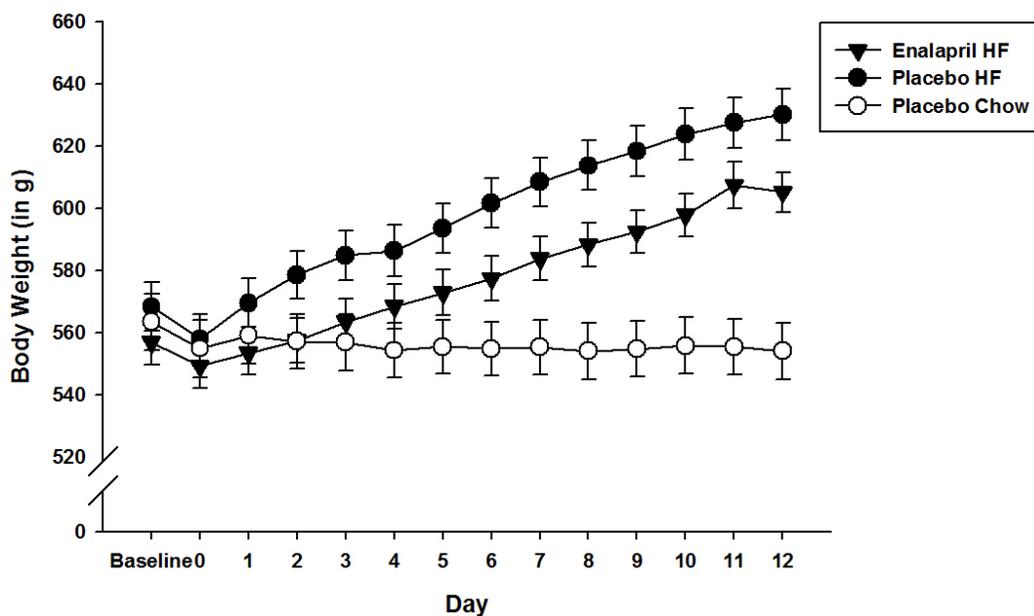


Figure 3-4. Daily change in body weight over the thirteen day high fat diet. Baseline values indicate the average weight of each group before the start of the diet. Error bars signify the SEM, or standard error of the mean, for each value.

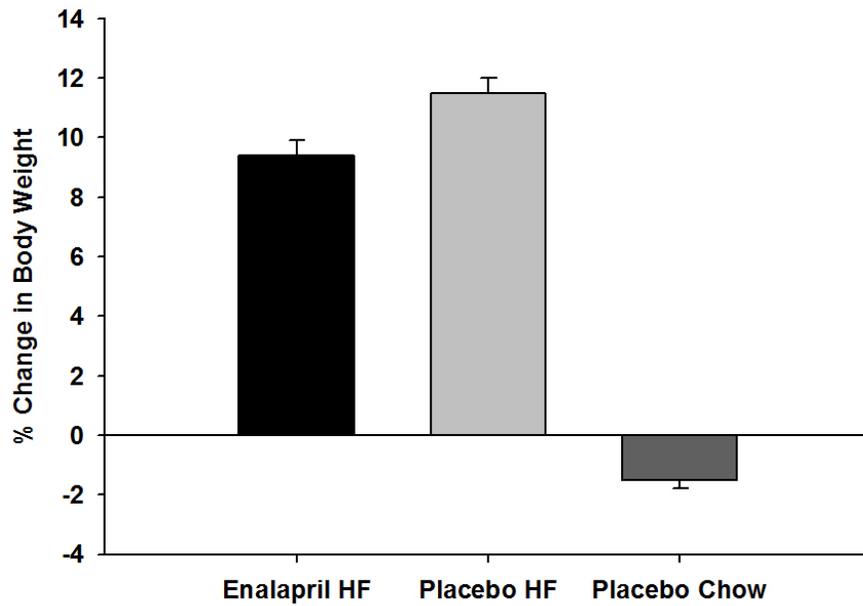


Figure 3-5. Percent change in body weight from baseline to the end of the high fat diet. Error bars signify the SEM, or standard error of the mean, for each group.

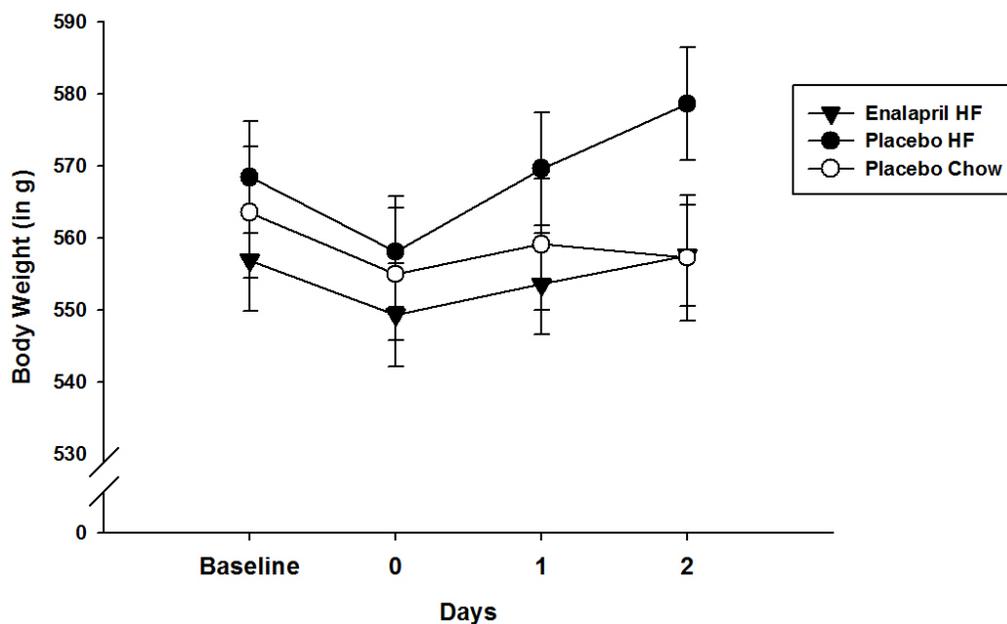
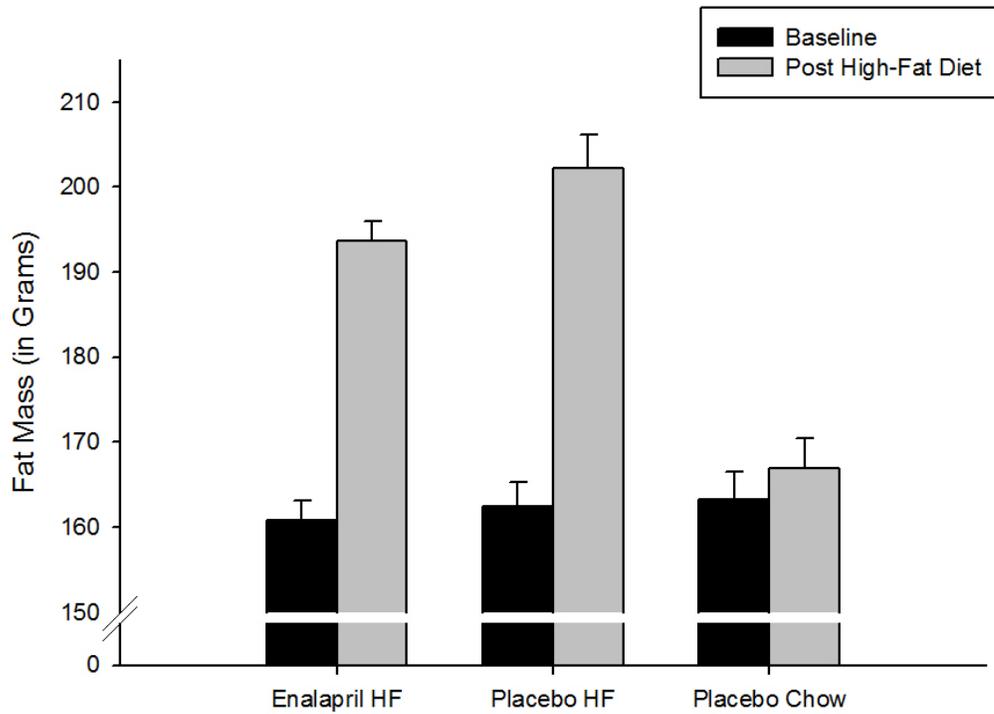
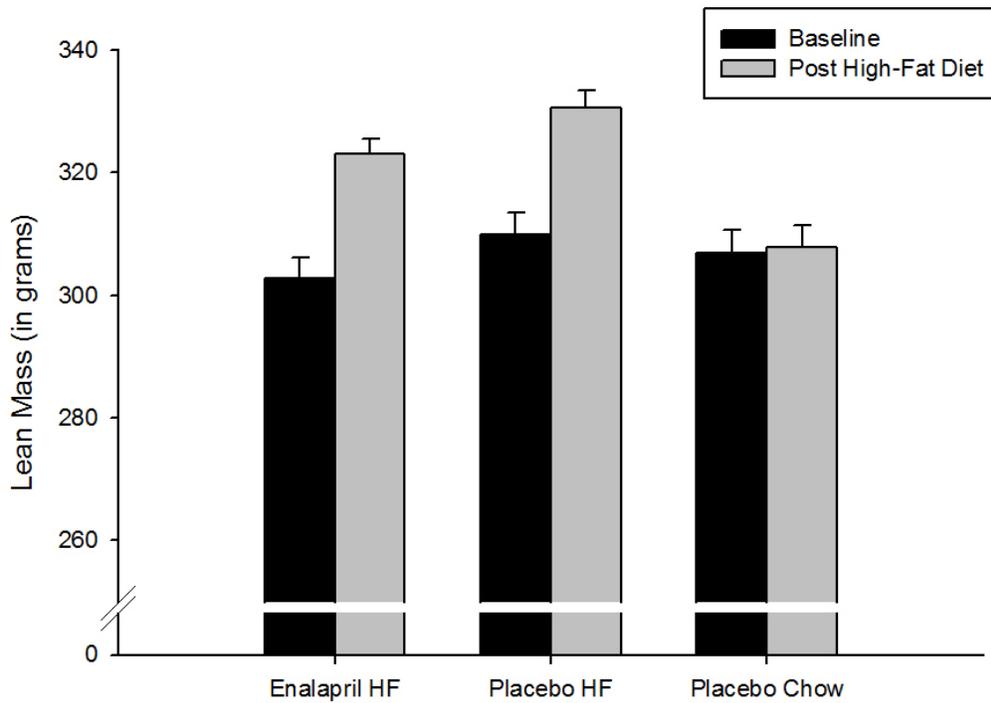


Figure 3-6. Change in body weight over the first three days of high fat diet. The data show the degree of weight gain due to hyperphagia. Error bars signify the SEM, or standard error of the mean, for each value.



A



B

Figure 3-7. Average fat and lean mass at baseline and post high-fat diet. Error bars signify the SEM, or standard error of the mean, for each value. A) Change in fat mass. B) Change in lean mass.

CHAPTER 4 DISCUSSION

Study Implications

We are witnessing a rise in the number of individuals at risk for physical disability due to the aging of the global population and a growing number of obese aged individuals. As aged obese individuals are more susceptible to chronic disease and disability, it is essential that we develop late-life pharmacological interventions. Numerous studies have demonstrated that enalapril is effective in mitigating body weight and fat tissue gain in aged, standard chow-fed rats. Thus, the goal of the present study is to determine if enalapril is efficacious in moderating food intake and subsequent weight and adipose gain in aged, high-fat fed rodents.

The results from our study show that the placebo HF group demonstrated a marked increase in food intake and experienced its largest weight gain on the first day of the high fat diet. In contrast, the enalapril HF group steadily increased consumption during the hyperphagic period, and did not peak until day 4. Unlike the placebo HF group, which experienced rapid weight gain after the first day of the diet, the enalapril HF group demonstrated a more moderate, consistent increase in weight over the first three days of the diet, closely mirroring the pattern of food intake. In addition, we observed that the pretreatment effects of enalapril on food intake and body weight disappeared after the first three days of the diet. Although consumption and body weight gain were significantly different between the placebo HF and enalapril HF groups during the hyperphagic period, these groups followed a similar pattern in weight gain and consumed equivalent amounts of food after this period. We predict that continuous enalapril treatment would have maintained significant differences in food intake and

weight gain throughout the high fat diet. Although the placebo HF and enalapril HF groups were gaining equivalent amounts of weight after the hyperphagic period, the placebo HF group weighed 21 grams more on average than the enalapril HF group and exhibited a 2.1% greater increase in body fat. The rapid weight gained experienced by the placebo HF group during the hyperphagic period can likely account for the larger overall increase in body weight and adiposity. It appears that the placebo HF group could not recover from the initial surge in food intake and subsequent weight gain, in spite of the fact that the placebo HF group's food intake was equivalent to that of the enalapril HF group by the seventh day of the diet. Upon initiation of the high fat diet, the high-fat fed animals discontinued eating their drug pellets, which may account for the plateau in food consumption between days 3 and 4. The data indicate that enalapril may attenuate weight gain by mediating the hyperphagic response to high-fat chow, although the precise mechanism by which enalapril exerts its effects is currently unknown.

Researchers in other labs have shown that enalapril treatment may be effective in improving insulin signaling. For example, treatment of the Cohen-Rosenthal diabetic hypertensive rodent strain with enalapril was effective in significantly lowering blood glucose levels (Rosenthal et al., 1997). However, previous studies in our lab have shown that enalapril does not ameliorate age-related insulin dysfunction in skeletal muscle nor whole body glucose tolerance in aged, chow-fed animals. 27 month old rats treated with 20 mg/kg/day of enalapril for three months did not experience improved glucose tolerance nor an increase in GLUT4 or AKT1 levels in the extensor digitorum longus (EDL) muscle, two markers of insulin signaling. Thus, the moderate weight loss induced by enalapril treatment may not be adequate for improving insulin function.

Since enalapril is ineffective in improving insulin function during normal aging in standard chow fed rodents, it is unlikely that enalapril will have any insulin-enhancing effects in the presence of high-fat feeding. A 2009 study by Santos evaluated the effects of enalapril in high-fat fed young rodents (Santos et al., 2009). As we observed in older rodents, enalapril treatment had no effect on the serum insulin levels in either the high fat or standard chow fed young animals. Although it is unlikely that insulin-dependent pathways are responsible for the observed effects of enalapril on high-fat fed aged animals, the SOCS-3 and leptin-signaling pathway have the potential to elucidate enalapril's mechanism of action.

It is possible that enalapril treatment may induce changes in leptin sensitivity. Leptin, a hormone secreted by adipocytes, functions in regulating energy expenditure and appetite. Circulating leptin is proportional to the quantity of adipose tissue, and exerts its effects on hypothalamic neurons in order to maintain body composition and nutritional status (Ahima & Lazar, 2008; Cohen et al., 2001). Leptin resistance is an inherent part of the aging process in both rodents and humans, and may contribute to development of age-related obesity (Zhang & Scarpance, 2006). Since leptin signaling plays a critical role in the regulation of food intake and body composition, it is possible that enalapril ameliorates hyperphagia by acting on the leptin signaling pathway. Peripheral and central leptin resistance are thought to contribute to the pathogenesis of age-related obesity. Human studies have shown that elevated leptin levels are ineffective in mitigating obesity, and leptin therapies are virtually futile in obese subjects (Zhang & Scarpance, 2006). Studies in rodents support the idea that age-related leptin-

insensitivity may exacerbate obesity in the elderly. Therefore, what follows is a review of the role of leptin in the regulation of body composition in the context of obesity.

Regulatory Role of Leptin in the Maintenance of Body Composition

Leptin, a product of the obesity gene (Ob gene) is an adipokine that communicates with the central nervous system, or CNS, regarding energy stores and nutritional state. The stomach, intestines, and skeletal muscle produce low levels of leptin, while adipose tissue produces the greatest concentration of leptin (Ahima & Lazar, 2008). Circulating leptin is proportional to the quantity of adipose tissue, adipocyte size, and triglyceride content (Ahima & Lazar, 2008), and varies depending on the abundance of certain adipose tissues. Fasting reduces circulating leptin levels, which in turn stimulates hyperphagia (increased feeding) and reduced energy expenditure. Conversely, overfed animals have a greater concentration of circulating leptin, resulting in reduced food consumption and increased energy expenditure (Ahima & Lazar, 2008). Leptin selectively targets adipose tissue and preserves lean muscle mass by inducing lipid oxidation and protein synthesis while reducing lipogenesis (Buettner et al., 2008). Studies in knockout mice have demonstrated leptin's critical role in maintaining nutritional state and body composition. Mice with leptin mutations (ob/ob) or leptin receptor mutations (db/db) demonstrate marked hyperphagia and resultant obesity. These mice also have concomitant metabolic and endocrine disorders, including diabetes, enlarged fatty livers, and hypercortisolemia, or the overabundance of cortisol (Cohen et al., 2001).

Leptin functions by binding to receptors in the hypothalamus and inhibiting appetite while promoting energy expenditure. Leptin receptors are present in peripheral tissues and throughout the CNS, but the highest concentration of leptin receptors

occurs in the arcuate nucleus of the hypothalamus (Banks, 2004). The leptin receptor has five splice variants (ObRa-ObRe); ObRb, the long form of the receptor, appears to be the isoform responsible for the neuroendocrine effects of leptin (Cohen et al., 2001). ObRb is found in peripheral tissues, and is enriched in the brainstem, the arcuate, ventromedial, dorsomedial, and paraventricular hypothalamic nuclei, and cerebral regions that manage food intake, metabolism, and regulate neuroendocrine systems (Ahima & Lazar, 2008; Cohen et al., 2001). Absence of ObRb produces a phenotype that is virtually identical to that observed in leptin-deficient animals, which elucidates the critical role of this receptor for body weight regulation (Cohen et al., 2001).

Leptin signaling involves several pathways, but the Janus tyrosine kinase 2 (JAK2/STAT3) pathway is the most well-understood. The intracellular domain of the leptin receptor contains three tyrosine residues: LepR^{y985}, LepR^{y1138}, and LepR^{y1077}. Phosphorylation of tyrosine 985 is responsible for negative feedback regulation and reduces leptin signaling; phosphorylation of tyrosine residue 1138 is required for phosphorylation and recruitment of STAT3 (Donato, Jr. et al., 2010). Leptin binds to the OBRb receptor in the hypothalamus, initiating phosphorylation and activation of JAK2, which is constitutively attached to ObRb. Phosphorylation of Tyr1138 recruits STAT3 to the ObRb-JAK2 complex. JAK2 phosphorylates STAT3 (p-STAT3), resulting in subsequent dimerization and translocation to the nucleus, where the STAT3 dimers promote gene transcription of pro-opiomelanocortin (POMC) and suppressor of cytokine signaling-3 (SOCS-3) (Donato, Jr. et al., 2010; Zhang & Scarpace, 2006; Munzberg & Myers, Jr., 2005). STAT3 signaling is critical for the long-term regulation of food consumption, body weight, and glucose homeostasis, and does so by regulating SOCS-

3. SOCS-3 binds to Tyr 985 of the leptin receptor and impedes JAK activity, attenuating leptin-receptor signaling. Studies have shown that SOCS-3 expression is elevated in obese rodents, and deletion of SOCS-3 in hypothalamic neurons enhances leptin sensitivity and minimizes susceptibility to diet-induced obesity (Donato, Jr. et al., 2010; Munzberg & Myers, Jr., 2005).

Since SOCS-3 contributes to leptin sensitivity, it is possible that enalapril may exert its effects by acting directly on SOCS-3 expression and signaling. Suppressors of cytokine signaling (SOCS) are essential regulators of cytokine activity and contribute to the regulation of inflammatory systems. SOCS-3 expression is induced by numerous anti-inflammatory and inflammatory cytokines and acts in a negative-feedback loop with the JAK-STAT pathway. SOCS proteins are normally expressed at low levels, but cytokines and hormones that activate JAK-STAT signaling also induce the rapid expression of SOCS proteins, thus inhibiting the continued activation of this pathway (Hanada et al., 2003; Muscogiuri et al., 2008). The increased expression of TNF- α and insulin in obese animals has been found to induce SOCS-3 mRNA expression in the white adipose tissue of obese mice (Emanuelli et al., 2001). In addition, Ang II has been found to induce SOCS-3 expression in both cultivated cell-lines and mammalian tissues (Muscogiuri et al., 2008). Thus, enalapril may exert its physiological effects by reducing circulating levels of Ang II, subsequently reducing SOCS-3 expression and increasing leptin sensitivity.

POMC produces the anorexic neuropeptide α -melanocyte stimulating hormone (α -MSH), which activates melanocortin-3 and -4 receptors in order to reduce food intake, body weight, enhance energy expenditure, and regulate glucose metabolism

(Niswender et al., 2004; Munzberg & Myers, Jr., 2005; Belgardt & Bruning, 2010). Agouti-related protein (AgRP) neurons inhibit melanocortin -3 and -4 receptor signaling and directly inhibit POMC neurons by release of γ -aminobutyric acid (GABA) to promote feeding and reduced energy expenditure. Neuropeptide Y (NPY), an orexigenic, or appetite-stimulating, hormone increases food intake and suppresses thermogenesis by releasing GABA and inhibiting POMC activity. ObRb is located in the ARC anabolic neuron populations responsible for production of AgRP and NPY. The interaction of leptin with its receptors inhibits expression of both AgRP and NPY, allowing for increased metabolism and reduced appetite (Niswender et al., 2004; Stephens et al., 1995; Munzberg & Myers, Jr., 2005; Belgardt & Bruning, 2010).

Although the JAK2/STAT3 pathway is the best understood and is necessary for the long-term effects of leptin, the phosphatidylinositol 3-kinase, or PI3K pathway is responsible for the leptin's acute effects. PI3Ks are heterodimeric complexes that contribute to regulation of metabolism, growth, and food intake. Activated JAK2 phosphorylates IRS proteins, which subsequently recruit PI3K in hypothalamic neurons and activates the PI3K pathway in POMC neurons (Niswender et al., 2004; Donato, Jr. et al., 2010; Munzberg & Myers, Jr., 2005). The specific neuron populations responsible for the effects of the PI3K pathway have not been completely identified. However, we know that the leptin-induced PI3K pathway has been correlated with maintaining glucose homeostasis in peripheral tissues and regulating the rapid effects of leptin signaling, such as diminished food intake (Belgardt & Bruning, 2010).

Obesity and the Development of Leptin Resistance

In young humans, there is a significant correlation between adiposity and plasma leptin. However, this correlation is lost in middle-aged and elderly individuals, which is indicative of dysregulation of leptin signaling (Unger, 2002). Although relative fat mass between young (20-40 y.o.) and older (> 40 y.o.) individuals may be comparable, leptin levels in older subjects are not correlated with fat mass. This reduced correlation is indicative of a feedback disruption between peripheral adipose stores and appetite regulation in the central nervous system of older individuals. It is hypothesized that the impaired feedback may be related to reduction of sex-hormones in older individuals and contributes to age-related obesity (Moller et al., 1998).

Studies in obese mice and humans reveal that despite elevated leptin, appetite is no longer suppressed, an indicator of leptin resistance. Although the precise mechanism of leptin resistance is currently unknown, the two main hypotheses include failure of circulating leptin to cross the blood-brain barrier (BBB) and reach its receptors in the hypothalamus, and defects in leptin-receptor signaling cascade (Kershaw & Flier, 2004; Munzberg & Myers, Jr., 2005). Leptin crosses the BBB by a saturable transport system which appears to have decreased activity in overnourished, obese rodents. The transport rate of leptin across the BBB can be attributed in part to self-inhibition of increased serum leptin concentration in obese animals (Munzberg & Myers, Jr., 2005; Banks, 2004). Brain perfusion studies demonstrate that the BBB is most efficient when circulating leptin levels are below normal levels. Leptin concentrations above 10 ng/mL, a level observed in obese animals, exceed the BBB transport threshold. As a result, as serum leptin concentration increases, the percentage of leptin transported across the BBB is diminished. Leptin levels of 30 ng/mL resulted in transport that was only 66% of

the value that was transported at 10 ng/mL. Elevated triglycerides is likely a significant contributor to BBB transport defects; both starvation and obesity result in elevated triglyceride levels, which inhibits leptin transport across the BBB. Inhibition of leptin transport is necessary during periods of starvation, as it prevents leptin from exerting its appetite-suppressing and metabolism-enhancing effects. However, elevated triglycerides in obese animals may be misinterpreted as starvation, so obesity is exacerbated since leptin is unable to interact with its receptors in the hypothalamus (Banks, 2004). It is thought that BBB resistance occurs before central resistance; animals with only a BBB transport defect respond to central administration of leptin, but do not respond to peripheral administration. However, prolonged obesity likely results in the development of central leptin resistance, subsequently disrupting the leptin signaling cascade (Kershaw & Flier, 2004). Studies in mice fed either a low- or high-fat diet show that leptin resistance initially develops with BBB transport defects, and eventually develops into central leptin resistance. Mice were evaluated with peripheral (ip) or central (icv) leptin injection after four weeks and fifteen weeks of a low or high fat diet. EMSA data revealed that ip injection induced equivalent STAT3 DNA binding in both diet groups after 4 weeks. However, ip injection was ineffective in inducing STAT3 binding in the high fat group after 15 weeks, indicating that BBB transport was compromised and subsequently prevented activation of STAT3 by leptin. Although icv injection induced STAT3 activation in high-fat fed mice after 15 weeks, induction was 75% lower than in low-fat fed mice, which indicates defects in the leptin-receptor signaling cascade (El-Haschimi et al., 2000).

In several rodent models of obesity, SOCS-3 expression has been elevated. When circulating leptin levels are low, baseline STAT3 activation is moderate, which in turn depresses SOCS-3 expression. As leptin concentration increases incrementally, leptin-receptor signaling rises in proportion to leptin levels. However, when leptin concentration is chronically elevated, baseline STAT3 activity and SOCS-3 expression is augmented, which diminishes the effects of increased leptin receptor activity. Adipose tissues secrete fatty acids and inflammatory cytokines such as IL-6 and TNF- α , which may enhance SOCS-3 expression (Munzberg & Myers, Jr., 2005). In addition, C-reactive protein (CRP), which is secreted by the liver and elevated in inflammatory states, has been found to bind to leptin and repress its interaction with ObRb. Not only does CRP inhibit leptin-receptor activity, but leptin itself induces hepatic CRP expression, exacerbating the leptin-resistant state (Belgardt & Bruning, 2010).

In a study conducted by Judge et al. (Judge et al., 2008), osmotic minipumps containing murine recombinant leptin (i.e. peripheral leptin infusion) were implanted in a subcutaneous pocket in both young (3 month old) and aged (30 month old) F344xBN rodents and the animals were infused with varying concentrations of leptin. The young rats demonstrated an anorectic response and reduction in body weight, and reduction in kilocalorie consumption and weight loss was dose-dependent, with the response plateauing at 0.07 mg/day. The aged rodents showed no response to leptin infusion, even at the maximum dose of 0.5 mg/day. The aged animals did not reduce caloric intake or lose weight, indicating a resistance to the effects of leptin. Upon initiation of the palatable, high-fat diet, the young rats spontaneously divided into either a diet-induced obesity (DIO) or diet-resistant (DR) groups. However, the high-fat fed aged rats

did not divide into these groups, and instead exhibited vast increases in weight. Typically, animals provided high-fat chow demonstrate hyperphagia upon initiation of the HF diet. In the young rodents, leptin-receptor activity allowed for normalization of caloric intake, and these animals return to pre-HF consumption levels. However, in the leptin-resistant aged rats, there was a delayed normalization of caloric intake, resulting in an extended hyperphagic effect that exaggerated increases in weight and adipose tissue in addition to a peak kilocalorie intake that was much greater than the peak intake observed in the young rats (Judge et al., 2008).

Enalapril as a Modulator of Leptin in the Context of Obesity

Studies conducted by Santos et al. (Santos et al., 2009) evaluated the effect of enalapril on both serum leptin concentration and leptin response in young rodents fed standard or high fat chow. The first study involved measuring serum leptin after young rats were fed either standard or hyperlipidic chow and simultaneously treated with 10 mg/kg/day of enalapril for six months. The data show that standard and high-fat fed animals treated with enalapril had lower serum leptin concentration compared to their placebo-treated counterparts. The decrease in leptin can likely be attributed to the reduced fat mass in enalapril-treated animals, as leptin concentration is proportional to adiposity.

The second study evaluated enalapril's effects on leptin response in the presence of a hyperlipidic diet. Intracerebroventricular (icv) leptin cannulas were implanted in three-month old male Wistar rats. The rats were fed either a standard or high fat diet in conjunction with 10 mg/kg/day enalapril treatment (dissolved in the animals' drinking water) for one month. 24 hours after injection, food intakes were recorded. Food intake was significantly reduced in the icv-treated diet groups, although there was no

significant difference observed between the enalapril and control groups. Lastly, leptin transport across the BBB was evaluated in a subset of these groups by intravenous injection of radioactively labeled leptin (^{125}I -leptin). The data show that enalapril did not alter transport of ^{125}I -leptin across the BBB (Santos et al., 2009).

Future Directions

The studies conducted by Santos and Scarpace provide an excellent basis for investigating the potential role of enalapril on leptin signaling in high-fat fed aged rodents. Judge et al. (Judge et al., 2008) demonstrated that aged rodents are leptin resistant and exhibit hyperphagia and delayed normalization of caloric intake when presented with hyperlipidic food. Santos et al. showed that icv administration of leptin effectively reduced food intake in the young high-fat fed rodents, although there was no significant difference between the control and enalapril-treated animals. In addition, enalapril did not increase BBB transport of leptin. Although enalapril did not exert significant effects in icv-treated animals or increase leptin transport, we must consider that the studies were conducted using a very low dose of enalapril (10 mg/kg/day), as opposed to the standard dose of 20-40 mg/kg/day used in our studies. Thus, it is quite possible that enalapril can alter the leptin signaling pathway in high-fat fed rodents, but the 10 mg/kg/day dose is too low to exert significant biological effects. As previously discussed, leptin concentrations above 10 ng/mL, a level observed in obese animals, exceed the BBB transport threshold (Unger, 2002; Banks, 2004). Therefore, enalapril may improve leptin transport by reducing overall adiposity and subsequently reducing circulating levels of leptin.

Our present study has shown that enalapril alone, at a dose of 40 mg/kg/day, is effective in reducing hyperphagia in high-fat fed aged animals. These findings warrant a

future investigation of the combined effects of leptin and enalapril in high-fat fed aged animals, and the potential effects on leptin transport. Since the animals ceased consumption of their drug pellets in our present study, our future study would involve administering 40 mg/kg/day of enalapril in drinking water so that the animals continue drug treatment while on the high-fat diet, potentially resulting in a more dramatic reduction in hyperphagia. ICV leptin cannulas would be implanted in half of the enalapril HF and placebo HF animals in order to determine if enalapril and leptin produce an additive effect in reducing hyperphagia. A subset of the enalapril HF and placebo HF groups would be injected with ¹²⁵I-leptin in order to evaluate whether enalapril improves leptin transport across the BBB in aged animals.

Since our target human population includes obese aged individuals, we aim to determine if enalapril will be effective in mitigating weight gain in those who are already in an obese state. Our present study investigated enalapril's effects in normal-weight rodents who were pre-treated with enalapril before introduction to high-fat food. Thus, we observed enalapril's effects before the onset of obesity. A future research aim will be to high-fat feed rodents first, followed by enalapril treatment when the animals are in an obese state. Thus, pre-treatment with a high-fat diet may provide a more realistic rodent model of our target human population.

Future experiments may also include those that focus on antagonism of various pathways. In vivo, nitric-oxide synthase (NOS) inhibition in rats with the ubiquitous NO blocker L-NAME results in reduced skeletal muscle, cross-sectional area, and subsequent disruption in walking speed (Wang et al., 2001). NO exists in three isoforms: endothelial NOS (eNOS), neuronal/mitochondrial NOS (n/mtNOS), and

inducible NOS (iNOS) (Dirks & Leeuwenburgh, 2005; Marzetti et al., 2010; Tracy et al., 1999). A low steady-state level of NO, which is maintained by constitutively expressed eNOS and nNOS, favorably modulates cellular metabolic function. In fact, low steady-state concentration reversibly inhibits cytochrome c release from mitochondria and subsequently reduces apoptosis (Beltran et al., 2000). iNOS, however, produces large and potentially toxic levels of NO and is increased with age.

Thus, we investigated enalapril's effects on NO production, based on the hypothesis that enalapril would increase production of eNOS and nNOS by reversing the proteolytic degradation of bradykinin. Subsequently, we predicted that increased production of the favorable isoforms of NO would preserve cellular metabolic function and improve skeletal muscle function. Male F344 x BN rats were treated with 20 mg/kg enalapril, 1 mg/kg L-NAME, a combination of enalapril and L-NAME, and placebo between 24 and 27 months of age. Evaluation of the TA muscle revealed that NOS activity was increased by enalapril treatment. However, L-NAME in combination with enalapril reduced NOS activity and also reversed the decrease in body weight that is observed with enalapril treatment alone. Therefore, we may use these data as a rationale for an in-depth evaluation of the bradykin pathway's effects on skeletal muscle function and body weight. Bradykinin blockers such as HOE-140, a highly potent B₂-receptor antagonist with a long duration of action (Bao et al., 2000), may be used to address these issues.

If our future studies establish that enalapril significantly improves leptin signaling and subsequently reduces hyperphagia and weight gain in high-fat fed aged animals, we will have a potential pharmacological intervention for effectively combating age-

related obesity in humans. Consequently, we may observe a reduction in the pathologies associated with age-related obesity, including metabolic dysregulation and inflammation. These health improvements in elderly individuals may reduce health care costs due to repeated hospitalizations, and potentially allow for increased independence and prolonged lifespan. Thus, it is imperative that we thoroughly investigate enalapril's full potential as a pharmacotherapy agent.

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