REDUCED ADIPOSE TISSUE HYPOXIA AS A POTENTIAL MECHANISM BY WHICH EXERCISE AND/OR LOW FAT DIET REDUCES INFLAMMATION IN OBESE MICE

BY

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THESIS

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Abstract

Recent evidence from our lab suggests that, in high fat diet-induced (HFD, 45% fat) obese mice, moderate exercise, low fat diet (LFD, 10% fat), or their combination results in significant reductions in the visceral adipose tissue inflammation. Adipose tissue hypoxia, perhaps involving cell stress or death, has been suggested as one of the major initiating events inciting inflammation. Purpose- The purpose of the proposed study was to investigate adipose tissue hypoxia as a potential mechanism by which exercise and/or low fat diet might exert anti-inflammatory effects in adipose tissue. Methods- Male C57BL/6 mice (n=73) fed a 45% high fat diet for 6 weeks to induce obesity were randomly assigned to one of 4 groups: exercised (5 days/week, 40 min/day, 65-70% VO_2 max) high fat diet or low fat diet or sedentary high fat diet or low fat diet for 12 weeks in a 2 x 2 design. In a subset of these mice (n=32), adipose tissue hypoxia was measured in epididymal fat pads using pimonidazole hydrochloride (injected intraperitonealy at a concentration of 60 mg/g body weight) and detected via ELISA. Results-Exercise and diet interventions had similar effects at attenuating body weight and epididymal fat pad weight gain as compared to a recent study from our lab where mice underwent the same interventions. There was a significant ($F_{1,23}$=6.3; p=0.02) exercise x diet interaction such that adipose tissue hypoxia in the combined intervention was significantly less than all other groups with a tendency for low fat diet alone to be more efficacious than exercise alone. Conclusion-These circumstantial data suggest that adipose tissue hypoxia may be a potential mechanism by which both regular exercise and/or a low fat diet reduce adipose tissue inflammation and that exercise when combined with low fat diet provides a strong enough stimulus to reduce adipose tissue hypoxia.

ACKNOWLEDGEMENTS

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CHAPTER 1: INTRODUCTION

Obesity is becoming increasingly common and by now most health professionals are aware of the metabolic and cardiovascular risks associated with obesity. There are numerous health risks associated with obesity including an increased chance of cardiovascular disease, type II diabetes, depression, hypertension, and cancer (2,49). Due to the increased social and economic consequences obesity is having on our culture, understanding the causes of and underlying physiological mechanisms associated with obesity has become a priority in current healthcare research.

Obesity is now viewed as a state of chronic inflammation with the immune system mediating many of the adverse metabolic effects of the disease. Recent evidence from our lab and others show that obesity is characterized by increased systemic inflammation (23) and increased local inflammation, in the adipose tissue itself (48,54,60). This is significant as increases in chronic inflammation are also linked to increased risk of a number of chronic diseases.

It is believed that adipose tissue hypoxia is likely one of the major initiating events inciting adipose tissue inflammation (50). In addition to an increased inflammatory response, adipose tissue hypoxia has also been linked to increases in macrophage infiltration, reduced adiponectin expression, increased leptin expression, increases in cell death, endoplasmic reticulum stress, and mitochondrial dysfunction (62).

It has also been well established that increased levels of physical activity are linked to reductions in chronic inflammation and reduced disease risk (59). Indeed, a recent study showed that increased physical fitness, even in the absence of body fat reduction, was associated with lower risk of mortality compared with those who had lower levels of fitness (42). However, the

mechanism by which exercise exerts its anti-inflammatory effect still remains in question. Recent work from our lab showed that both exercise, low fat diet, and their combination result in significant reductions of obesity-induced increases in inflammation, both systemically and locally in the white adipose tissue (52). One potential mechanism by which exercise may be reducing inflammation in the adipose tissue, which has not been examined and is investigated here, is by changes in adipose tissue hypoxia.

Purpose

It has been widely accepted that obesity is associated with low-grade inflammation and that adipose tissue hypoxia may contribute to this chronic inflammatory state. It has also been seen in many studies that increased levels of exercise and weight loss are associated with decrements in inflammation. The purpose of the proposed study is to investigate adipose tissue hypoxia as a potential mechanism by which exercise and/or a low fat diet might exert its anti-inflammatory effects in the adipose tissue of diet-induced obese mice. Adipose tissue hypoxia was measured in the epididymal fat pads via a chemical hypoxic probe, pimonidazole hydrochloride, a widely accepted marker of hypoxia.

Specific Aims

1. To investigate potential changes in adipose tissue hypoxia, as measured by pimonidazole hydrochloride concentration, in response to 12 weeks of exercise and/or low fat diet intervention in dietary-induced obese mice

2. To examine whether adipose tissue hypoxia plays a role in the reductions in adipose tissue inflammation recently found by our lab in response to such treatment

Hypothesis

1. Following 12 weeks of exercise training, diet-induced obese mice, regardless of diet, will exhibit reduced levels of adipose tissue hypoxia in epididymal fat pads compared to sedentary counterparts.

2. Both exercised and sedentary mice fed a low fat diet will see a reduction in adipose tissue hypoxia compared with counterparts fed a high fat diet.

3. Both exercise and a low fat diet will have unique independent and combined effects such that both treatments will reduce adipose tissue hypoxia.

CHAPTER 2: LITERATURE REVIEW

The Obesity Epidemic

Obesity is indisputably a condition of public health significance in the United States. Data from the 2003-2004 National Health and Nutrition Examination Survey (NHANES), using measured heights and weights, estimates that over 66% of all adults in the United States are classified as either overweight or obese and 33% are classified as obese. These statistics have risen dramatically from the 1976-1980 survey, in which just 47% were classified as overweight or obese with just 15% being classified as obese. This increase was not only seen in adults, but was also very pronounced in children. Between the two surveys, prevalence of overweight increased from 6.5% to 18.8% in children aged six to 11 years and from 5.0% to 17.4% in those aged 12-19 years (38).

These rising rates are a major concern for health professionals considering the risks that can be associated with obesity. In addition to increasing mortality from all causes, obesity is closely linked to hypertension, type 2 diabetes mellitus, dyslipidemia, gallbladder disease, osteoarthritis, coronary heart disease, stroke, sleep apnea, and other respiratory problems. Other studies have identified obesity as a risk factor for endometrial, breast, prostate, and colon cancer (2,49). These risk factors coupled with the increase in childhood obesity have led experts to predict that, for the first time in history, the current generation will have shorter life spans than their parents (39,57). These risks clearly indicate that obesity is a major public health concern.

As the prevalence of overweight and obesity has increased in the United States, so too has the related health care costs. A 1998 study by Wolf and Colditz (58) estimated the direct health care costs of obesity at $61 billion per year with an additional $56 billion in indirect costs.

Direct health care costs refer to preventative, diagnostic, and treatment services such as physician visits, medications, and hospital and nursing home care. Indirect costs are the value of wages lost by people unable to work because of obesity-related illness or disability, as well as the value of future earnings lost by premature death. Because the prevalence of overweight and obesity has increased since 1995, the costs today are even higher than these figures.

Causes of Obesity

Simply put, obesity is caused by an energy imbalance. That is, a surplus of energy consumed as calories in food versus energy expended through resting metabolism and physical activity. The current lifestyle has made this energy imbalance convenient by means of an increasingly sedentary lifestyle, along with an abundance of readily-available and appetizing foods high in calories and fat with much research linking consumption of high-fat foods to obesity (29). However, it is important to be aware that a diet resulting in any positive energy balance, regardless of macronutrient content, will result in fat accumulation and eventually lead to obesity.

One should also be aware that, not just the amount, but the type of fat consumed contributes to obesity. Evidence suggests that diets high in saturated fat are most likely to cause weight gain. Conversely, a diet high in mono and polyunsaturated fats including fish oils does not seem to cause obesity. In fact, people who consume diets high in fish have been known to be leaner and suffer from fewer cardiovascular diseases (13).

In addition to increasing food consumption, decreasing levels of physical inactivity have also contributed to the obesity epidemic with many taking on an increasingly sedentary lifestyle due to more sedentary jobs and an environment that discourages regular physical inactivity (19).

Physical inactivity contributes to weight gain via reductions in insulin and leptin sensitivity (4) and reduced caloric expenditure. Thus, it is not surprising that physical inactivity has been shown to be an integral part of weight loss with reports that over half of individuals who lose weight through diet alone eventually regain the weight they lost (14). Further, it is interesting to note that regular exercise, even in the absence of weight loss, is associated with a substantial reduction in total and visceral fat content in obese individuals and type 2 diabetics (35), proving that exercise can have beneficial effects without inducing weight loss.

Inflammation Background

Inflammation is the term used to describe the environment produced by activated macrophages, including an environment of chemokines, cytokines, and other proteins that are a central aspect of the innate immune response. This response may be acute, as in the case of physical injury or infection, or low grade and chronic, as in long-term infections and autoimmune diseases (26). Chronic, low-level inflammation is defined as a two- to fourfold increase in levels of circulating inflammatory and anti-inflammatory cytokines and acute-phase proteins such as C-reactive proteins along with minor increases in neutrophils and natural killer cells (6).

Cytokines

Cytokines are proteins made by cells that affect the behavior or other cells (26). Cytokines have many important immune functions and are involved in orchestrating the innate inflammatory response. Dysregulation of cytokine production, as occurs with ageing and in many chronic conditions, can have affects on immune function and disease resistance (17).

As mentioned, several chronic conditions are associated with shifts in cytokine profiles. For example, the proinflammatory cytokine tumor necrosis factor-α (TNF-α) is increased in a number of chronic conditions, such as obesity, atherosclerosis, and type 2 diabetes (9,22,24). Further, proinflammatory cytokines, such as TNF-α, have also been found to cause a state of dyslipidemia (9).

Macrophages: Mediators of Innate Immunity

Macrophages, which act as the mediators of innate immunity, are activated during an inflammatory response or are recruited to the host tissue from blood by chemokines and provide a first line of defense against a host of common microorganisms. Traditionally, macrophages have been considered cells that simply secrete inflammatory proteins and phagocytose pathogens. However, it is now understood that macrophages are much more complex.

Macrophages can either be classically or alternatively activated. In the classical response, macrophages are activated to kill bacteria in response to exposure to the cytokine interferon gamma or lipopolysacharide, which is used as an activator of the immune response. These cells are considered pro-inflammatory since they produce pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-12, and TNF-α (37). Conversely, macrophages can be alternatively activated by exposure to different cytokines, such as IL-4, IL-13, or transforming growth factor-beta. These macrophages are considered anti-inflammatory as they are known to secrete anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist. They also produce other factors that promote wound healing and repair, such as transforming growth factor beta and vascular endothelial growth factor (VEGF). These macrophages are also thought to

have a role in tissue healing and repair via the promotion of angiogenesis or the stabilization of atherosclerotic plaques (16).

Many studies, including some from our lab, have shown that, as a result of aging and disease, macrophage function is dysregulated. It has been suggested that this dysregulated macrophage function may lead to increased susceptibility to infection (7) and a reduced capacity for wound healing (36). To date, not much is known about the effects of exercise on alternative macrophage activation.

Obesity and Adipose Tissue Inflammation

Chronic low-grade inflammation is common in obesity, especially in the case of visceral adiposity. Obesity-associated inflammation is characterized by increased levels of inflammatory mediators both systemically, as measured in the plasma (23), and locally, in the adipose tissue (48,60,54).

A large part of this immune response involves the infiltration of macrophages into the visceral white adipose tissue (60,54). These attracted macrophages then release large amounts of pro-inflammatory cytokines such as TNF-α, causing the production of monocyte chemoattractant protein-1 (MCP-1), which further attracts more macrophages to the visceral adipose tissue, thus creating chronic inflammation and intensifying the inflammatory response (48). In vitro studies have shown that macrophages and adipocytes interact in a paracrine manner, with TNF-α secretion from macrophages interfering with adipocyte insulin signaling and inducing fatty acid lipolysis. These fatty acids intensify the inflammatory response of the macrophages, creating a fierce cycle of inflammation and insulin resistance (10). It is widely believed that adipose tissue

macrophage infiltration increases local inflammation, which leads to reduced insulin sensitivity (48).

Until recently, adipose tissue has been viewed as a relatively inactive depot simply used for excess dietary fat. Recent evidence, however, now points to adipose as being a very active tissue; in some cases, acting as an endocrine organ and releasing hormone-like soluble mediators known as adipocytokines, a term used to describe cytokines released by the adipose tissue (23). Several of these adipocytokines have a central role in the regulation of inflammation and immunity. Adiponectin, leptin, resistin, and visfatin are all adipocytokines thought to provide an important link between obesity, insulin resistance, and related inflammation. Other products produced by adipose tissue include cytokines such as TNF-α, IL-6, IL-1, and MCP-1. These are not normally classified as adipocytokines, but have well established roles in immunity and metabolism. In humans, adipocytokines function as both hormones, influencing energy homeostasis and regulating neuroendocrine function, and as cytokines, affecting immune functions and inflammatory processes throughout the body (23).

Exercises Reduces Inflammation

According to a recent review published by our lab (59), both epidemiologic and longitudinal studies suggest that increasing physical activity is an effective means of reducing systemic low-level inflammation in conditions such as obesity, metabolic syndrome, and diabetes, as well as in healthy aged individuals. This is important because inflammation has been found to be a contributor to many chronic diseases, such as cardiovascular disease, colorectal cancer, stroke, type 2 diabetes, chronic obstructive pulmonary disease, and Alzheimer's disease (44,45,55).

Indeed, epidemiological studies have shown that exercise is an effective way of preventing several chronic diseases (11,46). Some studies have also shown that physically active individuals are less susceptible than sedentary individuals to viral and bacterial infections (46,12), suggesting exercise likely improves overall immune function.

Several recent studies, including some from our lab, suggest that cardiovascular training in both human (41,53) and mouse (1,52) models of obesity exert anti-inflammatory effects. These effects are seen both systemically and in the adipose tissue itself. At least 17 cross sectional studies found that serum CRP, a marker of chronic inflammation produced by the liver, was lowest in the highest category of physical fitness and highest in the lowest category of physical fitness (32). Another cross-sectional study found that active subjects had significantly lower levels of the pro-inflammatory cytokine IL-6 and higher levels of anti-inflammatory cytokine IL-10 (27). Longitudinal studies seem to show the same effect as, with few exceptions, most indicate that exercise training has an anti-inflammatory effect for individuals who have chronic diseases such as heart disease and metabolic syndrome and for overweight but otherwise healthy children and adults (59). Collectively, studies seem to suggest an anti-inflammatory effect of exercise and the possibility of a dose response, such that increased fitness is correlated with an anti-inflammatory profile.

Another cross-sectional study showed that the inverse relationship between physical activity and inflammatory markers was virtually abolished when the authors adjusted for the effect of body mass index, suggesting that the anti-inflammatory effect of exercise may be mediated by a reduction in body fat (42). Other potential mechanisms thought to possibly mediate the anti-inflammatory effect of exercise include reduced macrophage accumulation,

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increased levels of IL-6 antagonizing the proinflammatory effect of TNF-alpha, and activation of the cholinergic anti-inflammatory reflex (59).

Adipose Tissue Hypoxia

The role of adipose tissue hypoxia in obesity was recently review by Ye (62) who explains that several signaling pathways have been proposed to explain the pathogenesis of the inflammation associated with obesity, such as activation of toll-like receptor 4 by fatty acids, activation of protein kinase C by fatty acid derivatives, induction of endoplasmic reticulum stress, and activation of macrophages by adipocyte death. As explained, white adipose tissue is inflamed and undergoes a number of metabolic and endocrine dysfunctions in the obese state. It has been shown that hypoxia occurs in the adipose tissue of obese mice in both genetic and diet-induced models of obesity with hypoxia being suggested as a potential risk factor or another possible mechanism for the chronic inflammatory state associated with obesity (21,61).

Trayhurn (50) first proposed hypoxia as a possible cause of the link between obesity and inflammation in 2004. However, little information arose for the next three years, likely because there was no established method for the measurement of hypoxia in the adipose tissue (62). Four different methods are now regularly utilized to measure adipose tissue hypoxia. The first is the direct measurement of the interstitial partial pressure of oxygen. This method is able to provide definitive information about oxygen level in the adipose tissue via the insertion of an optical oxygen probe. The second approach involves detection via a chemical hypoxic probe, pimonidazole hydrochloride, which reacts with proteins in a low-oxygen environment leading to the generation of new protein adducts. The probe can then be stained for and measured via western blot or enzyme-linked immunosorbent assay (ELISA). In the third technique, a group of

widely accepted hypoxia-responsive genes are used as markers of hypoxia. These genes most often include hypoxia inducible factor-1-α (HIF-1- α), VEGF, glucose transporter 1 (GLUT1), heme oxygenase 1(HO-1), and pyruvate dehydrogenase kinase-1 (PDK1). HIF-1- α is a transcription factor that controls the expression of the other four genes in response to hypoxia. The fourth technique is a lactate assay in the adipose tissue, with lactate being used as an indirect indicator of hypoxia (62).

Adipose Tissue Hypoxia and Inflammation

As Ye puts it (62), "adipose tissue hypoxia may provide an answer to the question about the cause of chronic inflammation in adipose tissue in obesity." Hypoxia is able to induce inflammation in adipose tissue by the induction of the expression of certain genes in adipocytes and macrophages as evident by studies using primary cells and cell lines (21,61). The induced genes include TNF- α, IL-1, IL-6, MCP-1, plasminogen activator inhibitor-1 (PAI-1), macrophage migration factor (MIF), inducible nitric oxide synthase (iNOS), matrix metalloproteinases 9 (MMP9) and MMP 2 while the molecular activation mechanism is related to the activation of both NF-kB and HIF-1- α (62).

Activation of the transcription factor NF-kB by hypoxia has been well established in the fields of cancer biology, immunology, and cardiovascular research (62). It is known that activation of NF-kB can lead to transcription of many pro-inflammatory cytokines (such as TNF-α, IL-1, and IL-6) and other inflammation mediators (62). The signaling pathway for NF-kB has been well documented (15,18) and will not be discussed.

It has been well documented that macrophages infiltrate obese adipose tissue with macrophage infiltration now being used as a marker of chronic inflammation in the adipose

tissue (54,60).   Generally, it has been accepted that macrophage infiltration occurs as a result of increases in MCP-1 (30,31), but it was recently reported that macrophage infiltration was not associated with MCP-1 in both lean and obese mouse models (8,25).

Adipose tissue hypoxia may provide new insight into the mechanism of macrophage infiltration into the adipose. Recent studies show that hypoxia increases the expression of MIF (61), which is known to be an inhibitor of macrophage departure (51). Since tissue macrophage content is a result of both increased arrival or decreased departure of macrophages, this leads to an increase in adipose tissue macrophages (62).

Adiponectin is an anti-inflammatory cytokine produced by adipocytes that is decreased in obesity. The cause of this reduction in adiponectin remains unclear. Hypoxia has been shown to reduce adiponectin expression in cell-cultured adipocytes (21,61). It has been hypothesized that adipose tissue hypoxia may inhibit adiponectin expression either directly or indirectly by acting through TNF-alpha, but this mechanism remains to be investigated (62).

In addition to the previously mentioned increases in pro-inflammatory cytokines and macrophage infiltration, and decreased adiponectin expression, adipose tissue hypoxia has also been linked to a number of other biological outcomes. These include increased expression of leptin (61,62), increased cell death (63), endoplasmic reticulum stress (21), and mitochondrial dysfunction/reduction (33).

Possible Causes of Adipose Tissue Hypoxia

Ye proposes a number of possible causes of adipose tissue hypoxia (62). One possibility may be related to the reduction in adipose tissue blood flow that is seen in both obese humans (28,47) and animals (3,56). This reduction in adipose tissue blood flow is associated with

increased insulin resistance in obesity (28). This association has been known in obesity research for quite some time, but the events linking the two have not been investigated. Therefore, hypoxia may serve as a potential link.

Reductions in capillary density may also contribute to adipose tissue hypoxia (62). This is supported by the fact that VEGF is not increased in response to hypoxia as other hypoxia-responsive genes are (61). Further, reductions in endothelial density are also seen in obese mice (40). Ye surmises that the angiogenesis is reduced in obese adipose tissue and that this defect may account for the reduced adipose tissue blood flow (62).

Another possible cause of adipose tissue hypoxia may be the increase in adipocyte size that is seen with obesity. The diffusion distance of oxygen is 100-120 μm at most (20). In the obese state, adipocyte size increases up to 140-180 μm (5). Therefore, this increased size may block diffusion with oxygen not being able to reach the cells outside the 120 μm range, causing hypoxia (63).

Adipose Tissue Hypoxia and Glucose and Lipid Metabolism

In addition to increases in inflammation, adipose tissue hypoxia has also been linked to defects in glucose and lipid metabolism. A recent study found that hypoxia led to decreased insulin sensitivity in the adipocytes in obese mice. These decrements were linked to reductions in insulin receptor substrate-1 and insulin receptor-beta. This same study found that, in response to hypoxia, adipocyte free fatty acid uptake was reduced and lipolysis was increased in adipocytes. The molecular mechanism of decreased fatty acid uptake seemed to be related to the inhibition of the fatty acid transporters FATP1 and CD36 (63).

Hypoxia Related Signaling and Angiogenesis

HIF-1- α is a master signal mediator of the response to hypoxia. HIF-1 is a heterodimer consisting of both an α and β subunit. The HIF-1- β subunit is constitutively expressed whereas HIF-1-α expression is up-regulated during hypoxia (65). HIF-1-α is degraded rapidly under normal oxygen conditions via the ubitquitin pathway, but remains stable under hypoxic conditions. When stabilized, HIF-1-α translocates to the nucleus and combines with HIF-1- β in the nucleus and transciptionally activates a number of genes (66). HIF-1- α has been shown to be increased in a number of obese animal models (62). Further, HIF-1- α was reduced in obese patients following surgery-induced weight loss (67). HIF-1- α activity can be measured via both gene expression by reverse transcription polymerase chain reaction and protein measurement by western blot.

Some target genes for HIF-1 include VEGF, plasminogen activator inhibitor-1 (PAI-1), and leptin (50). Indeed, hypoxia does lead to an induction of leptin and VEGF expression showing that hypoxia likely leads to the stimulation of angiogenesis in adipose tissue via HIF-1 (68). Angiogenesis refers to the growth of new blood vessels from pre-existing vessels. It is a normal physiological process and is vitally important to both growth and development. However, in the case of adipocytes, angiogenesis is strongly suspected of promoting tissue growth and the excessive adipose tissue development leading to obesity (68). In fact, anti-angiogenic treatments performed on a number of mouse models of obesity have resulted in significant body weight and adipose tissue loss (69). A summary of the events that take place following adipose tissue hypoxia can be seen in **figure 1**.
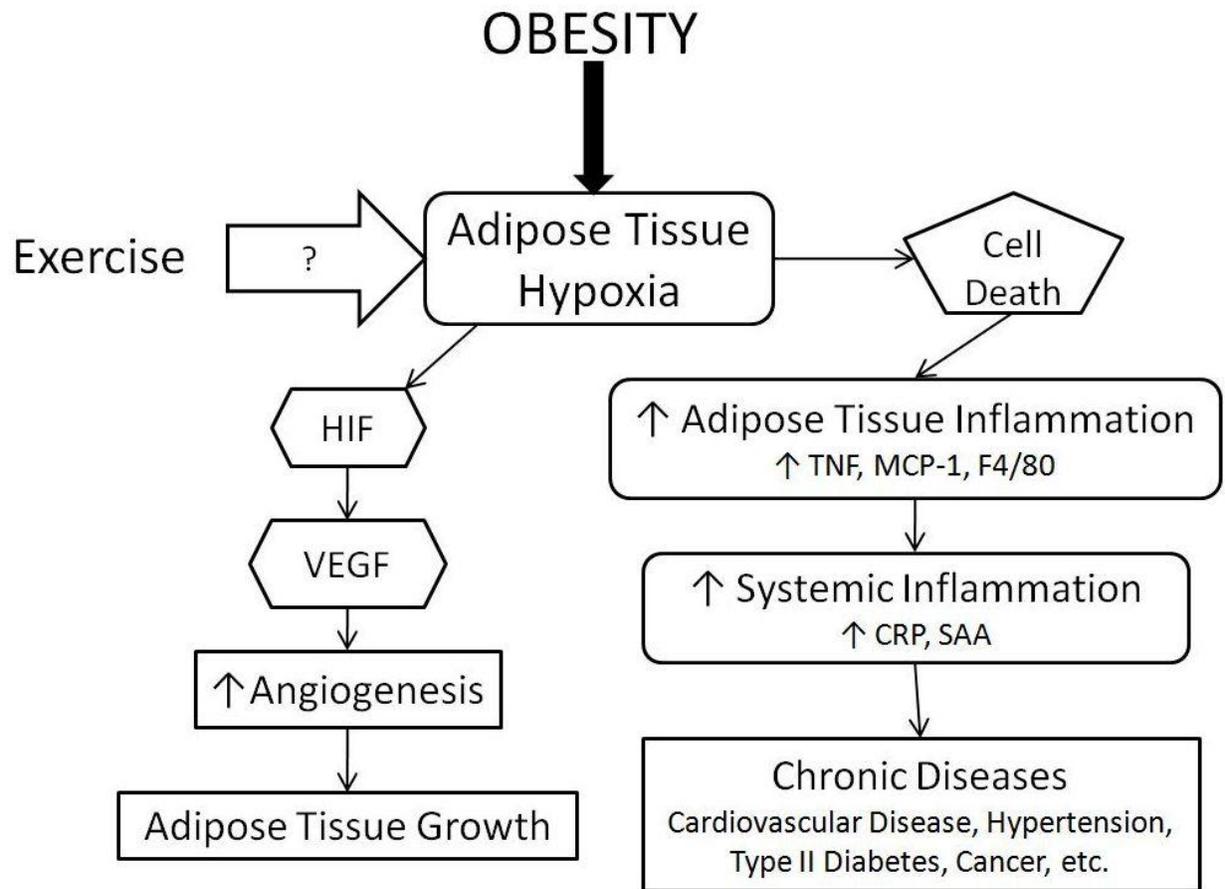
Figure 1 shows an overview of the events initiated following adipose tissue hypoxia in the obese state.

CHAPTER 3: METHODS

Study Design

This study addressed the effects of exercise and/or a low fat diet on adipose tissue hypoxia in obese mice and to see if these changes mediate reductions in adipose tissue inflammation. Animals used for the study were five week old male C57/BL mice (n=73) ordered from Jackson Laboratory. After approximately one week of acclimation, the mice were randomized into two groups. The first group (n=14) was placed on a CHOW diet served as controls for the entirety of the study. The second group (n=59) was placed on a high fat diet for six weeks (60% fat by kilocalorie). Following the six weeks of high fat feeding, mice were then randomized into four groups: high fat exercise, low fat exercise, high fat sedentary, and low fat sedentary. The low fat groups were placed on a low fat diet (10% fat by kilocalorie) while the high fat groups remained on the high fat diet. The exercise groups began moderate treadmill exercise for 40 minutes per day, five days per week while the sedentary groups remained inactive. Prior to intervention, following the initial six weeks of high fat feeding, mice underwent retro-orbital eye bleeds. The mice underwent their assigned intervention for 12 weeks and were sacrificed 24 hours following their last exercise session. Prior to sacrifice, a subset of mice (n=32) were injected with pimonidazole hydrochloride intraperitoneally at a concentration of 60 mg per g body weight. Tissues were harvested immediately after sacrifice, weighed, flash frozen and stored for later analysis. This paradigm, with the exception of the added control group, is similar to the model used in a recently published study by our lab and summarized in **figure 2** (52).
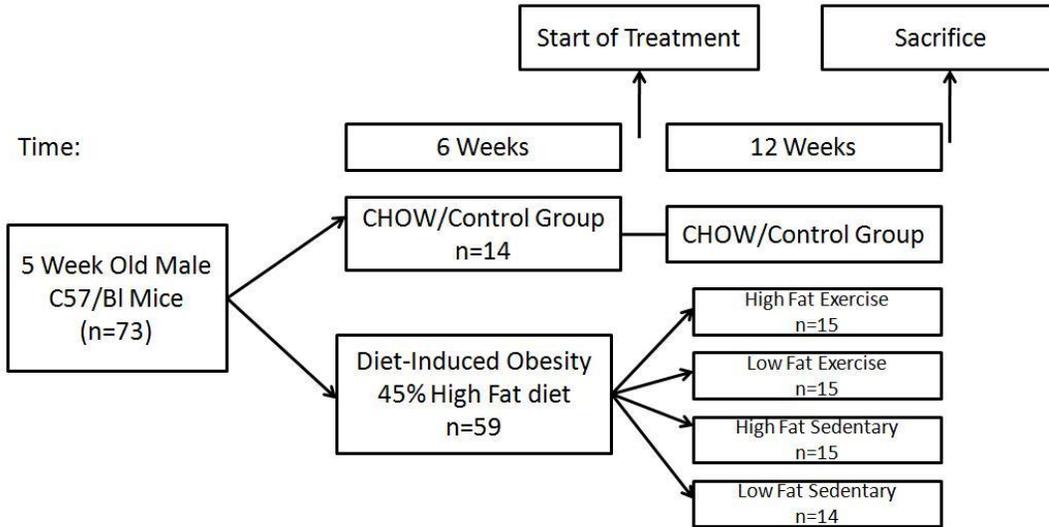
Figure 2 shows an overview of the study design

Animals and Diet

Male C57/Bl mice (n=73) were purchased from Jackson Laboratories (Bar Harbor, ME) at 4 weeks of age. In order to induce obesity, a well accepted model of diet-induced obesity was utilized. Mice were assigned to either the control or diet-induced obesity group. Control mice were fed a standard CHOW diet (n=15) while the diet induced obesity group was fed a 45% high fat diet for six weeks (n=58). This diet is well-tolerated and induces significant fat weight gain in the mice. For this experiment, obesity was induced by feeding mice for 6 wks on the diet and then randomizing them to one of four interventions: high fat sedentary, high fat exercised, low fat sedentary, or low fat exercised. Both the high and low fat diets were purchased from Research Diets Inc. (New Brunswick, NJ). The macronutrient content and main ingredients of both research diets are shown in **table 1**. All groups were allowed to eat ad libitum and food intake and body weight were recorded twice per week for the duration of the study. Strict

guidelines for the care and use of laboratory animals as directed by the National Institute of

Health were followed and all experiments were approved by the Institutional Animal Care and

Use Committee and supervised by the Division of Animal Resources at the University of Illinois

at Urbana-Champaign.

	High Fat Diet		Low Fat Diet	
Macronutrient	**% weight**	**% kcal**	**% weight**	**% kcal**
Protein	24	20	19.2	20
Carbohydrate	41	35	67.3	70
Fat	24	45	4.3	10
Ingredient	**Gram**	**% kcal**	**Grams**	**% kcal**
Sucrose	172.8	691	350	1400
Soybean Oil	25	225	25	225
Lard	177.5	1598	20	180
Total	858.15	4057	1055.05	4057

Table 1 shows the macronutrient distribution and main ingredients in both the high fat and low fat diets used

(Rodent diets D12450B and D12451, Research Diets Incorporated, New Brunswick, NJ)

Mouse Treadmill Running

Mice were exercise trained during their dark cycle (active period) between 09:00h and

12:00h on a motorized treadmill (Jog-a-Dog, Ottawa Lake, MI). Our lab has found that most

(~90%) mice are good runners and will keep speed with the treadmill with occasional gentle

prodding. Negative reinforcement (i.e. mild electrical shock) was never used as we have found

that it is unnecessary. Instead, foam padding was placed at the back of the treadmill lanes as

mice will respond to the tactile response. Mice were exercise trained at a moderate level defined

as brief (40 minute) bouts of exercise at a speed of 12 m/min at 5% grade five days per week.

The duration of exercise was increased gradually with mice starting off at 20 minutes per day

and increasing two minutes per day until 40 minutes was reached.

Retro-orbital method for blood collection

Following the six week time point, all mice were bled (~200µl) from the retro-orbital vein. Prior to collection of blood from the retro-orbital vein each mouse was administered isoflurane with oxygen at a flow rate of 2 to 3 liters per minute until unresponsive (approximately one to two minutes). This allowed sufficient time to complete the procedures while giving the mice the minimum amount of isoflurane needed. The mice were then restrained by the skin on the back of their neck and had the tip of a glass pipette inserted behind their left eye (~1-2mm deep). If necessary, each mouse then had a gauze pad placed over its eye to stop the bleeding and then was placed back in its cage and allowed to regain consciousness. Mice were observed until normal behavior was achieved (moving around cage, grooming, not bleeding from the eye) with most mice returning to normal behavior within five minutes of being returned to their cage.

Measurement of Adipose Tissue Hypoxia

Adipose tissue hypoxia was measured via hypoxyprobe administration in a subset of mice (n=25). Hypoxyprobe consists of two principal components: a small molecule hypoxia marker, pimonidazole hydrochloride, that selectively binds to proteins in oxygen starved cells, and a monoclonal antibody, hypoxyprobe-1-Mab1, that is used to detect pimonidazole protein adducts. One hour prior to sacrifice for tissue collection at the end of the study, mice were injected with pimonidazole hydrochloride intraperitoneally at a concentration of 60 mg per g body weight. Tissues collected at sacrifice allowed for tissue hypoxia quantification via measurement by enzyme-linked immunosorbent assay (ELISA). This approach has been used recently to measure adipose tissue hypoxia (63).

Hypoxyprobe ELISA

The ELISA kit used for Hypoxyprobe detection was developed by Raleigh et al. (70,71) and purchased from Hypoxyprobe, Inc (Burlington, MA). The kit contains three reagents: pimonidazole hydrochloride, hypoxyprobe-1 ficoll solid phase antigen, and a rabbit anti-hypoxyprobe-1 antiserum. The kit is a two-step competition assay.

Preparation of Tissue for ELISA Analysis

Previously frozen epididymal adipose tissue was put through a number of steps to prepare for hypoxyprobe ELISA detection. Tissue samples were weighed and added to PBS-tween at a ratio of 10 parts PBS-tween to 1 part tissue assuming 1 gram tissue is equivalent to 1 ml. The tissue was then homogenized with a mechanical homogenizer and a protein assay (described below) was carried out on this homogenate. The homogenized sample was then mixed with an equal volume of 1 mg/ml proteinase K in PBS-tween and placed overnight in a shaking water bath at 37°C. Proteinase K was then inactivated by adding a 20 millimolar stock solution of phenylmethylsulfonylflouride dissolved in dry dimethyl sulfoxide and incubated for five minutes at room temperature. This solution was then boiled for ten minutes in order to completely inactivate the protease which can interfere with the analysis of Hypoxyprobe antigen. These solutions were then centrifuged for 10 minutes at 10,000 repetitions per minute. The supernatant was then used for ELISA analysis.

Plate Preparation

ELISA plates were initially coated with a 1/500 dilution of solid phase antigen and incubated at 37°C for 1-2 hours. After washing with a phosphate buffered saline (PBS)-tween

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mixture, the plates were then blocked with a 1% gelatin solution in PBS-tween and incubated for 1-2 hours at 37°C for 1-2 hours. Following preparation, plates were stored at 4°C.

Pre-incubation

Standards of pimonidazole hydrochloride are diluted in a PBS-tween solution. For analysis, a linear standard curve was performed using dilutions between 2.5 and 0.02 micromolar. Prepared tissue samples were diluted four and eight fold in order to obtain concentrations that fell within this range. In each well of an assay plate, 100 microliters of standards and samples were added in duplicate followed by 25 microliters of diluted rabbit polyclonal, anti-hypoxyprobe antiserum and incubated at 37°C for one hour.

Competition Assay

The contents of each well of the assay plate were then transferred to the ELISA plate coated with the Hypoxyprobe solid phase antigen by means of a multiport pipette and incubated for one hour at 37°C. The contents were then decanted and washed four times with PBS-tween. Next, 100 microliters of a 1:2000 dilution of an alkaline phosphatase conjugated, goat anti-rabbit IgG antibody diluted in PBS-tween was added to each well and the plate incubated for one hour at 37°C under a cover of parafilm. The contents of the wells were decanted and the plate washed four times with PBS-tween. One hundred microliters of 1 mg/ml of alkaline phophatase substrate dissolved in 10% diethanlomine buffer was then added to each well of the ELISA plate. Color development was allowed to develop followed by an endpoint analysis of optical density at 405 nanometers. An example standard curve from one of the plates is shown below in **figure 3**. Notice that, due to the competitive nature of the assay, absorbance decreases as concentration increases as higher levels of hypoxyprobe lead to increased binding with the polyclonal antibody

in the preincubation step. This leads to less available polyclonal antibody to bind to the solid

phase antigen and subsequently leads to less binding with the alkaline phosphatase conjugated

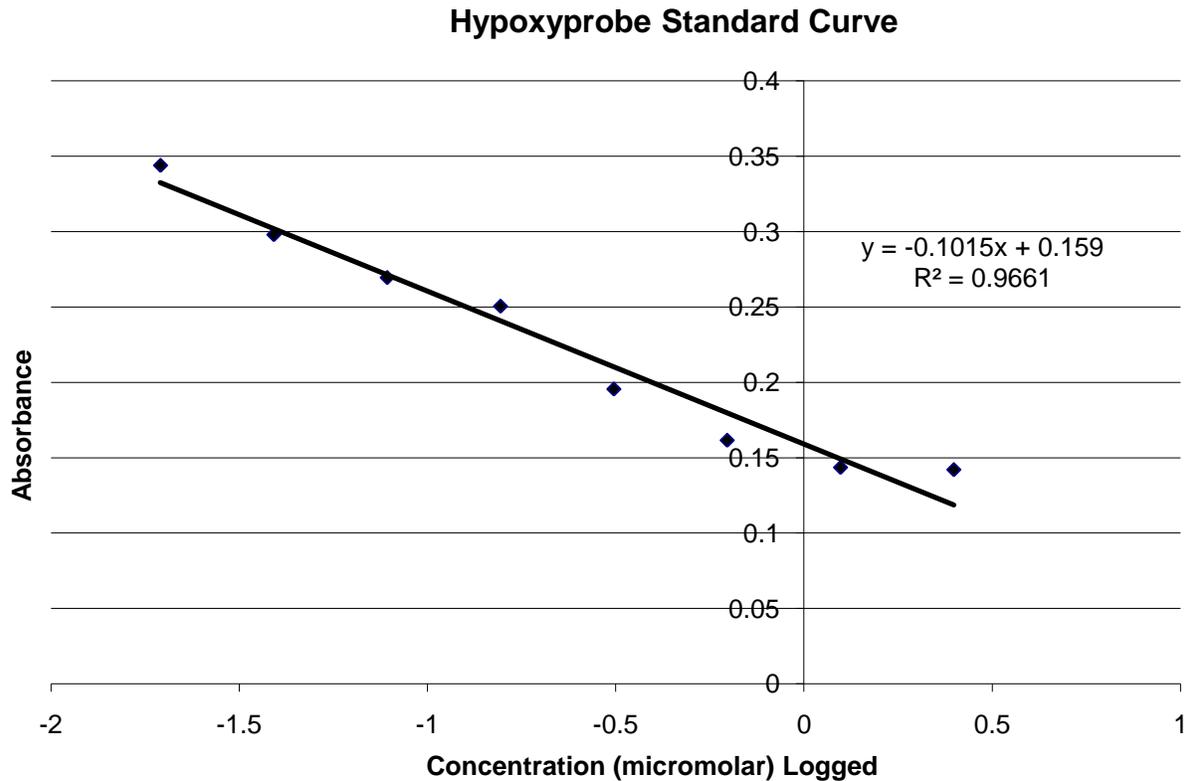antibody and less substrate binding and lower absorbance values.

Hypoxyprobe Standard Curve

$y = -0.1015x + 0.159$
$R^2 = 0.9661$

Figure 3 Example standard curve from hypoxyprobe ELISA kit. Note that concentration is logged and that as

concentration increases, absorbance decreases. The absorbency was read at 405 nm using standards from 2.5 to .04

micromolar.

Determination of Tissue Protein Content

As hypoxyprobe only infiltrates protein adducts, it is necessary to normalize

hypoxyprobe concentration to tissue protein content. In order to determine protein

concentration, a protein assay was carried out using the Bio-Rad DC Protein Assay Kit (Bio-Rad,

Hercules, CA). For analysis, a linear standard curve was prepared using concentrations between

0.125 and 2.0 mg/ml prepared using bovine serum albumin dissolved in PBS-tween. Five

microliters of standards and samples were then added to each well of an assay plate in duplicate.

Next, 25 microliters of working reagent A was added to each well. Working reagent A was

prepared by adding 20 microliters of reagent S to each milliliter of reagent A needed for the

assay. Two hundred microliters of reagent B was then added to each well. After shaking to mix

reagents, absorbances were read at 750 nanometers. An example of a standard curve is shown in

figure 4.

Bio-Rad DC Protein Assay Standard Curve

$y = 0.0867x + 0.0982$
$R^2 = 0.9979$

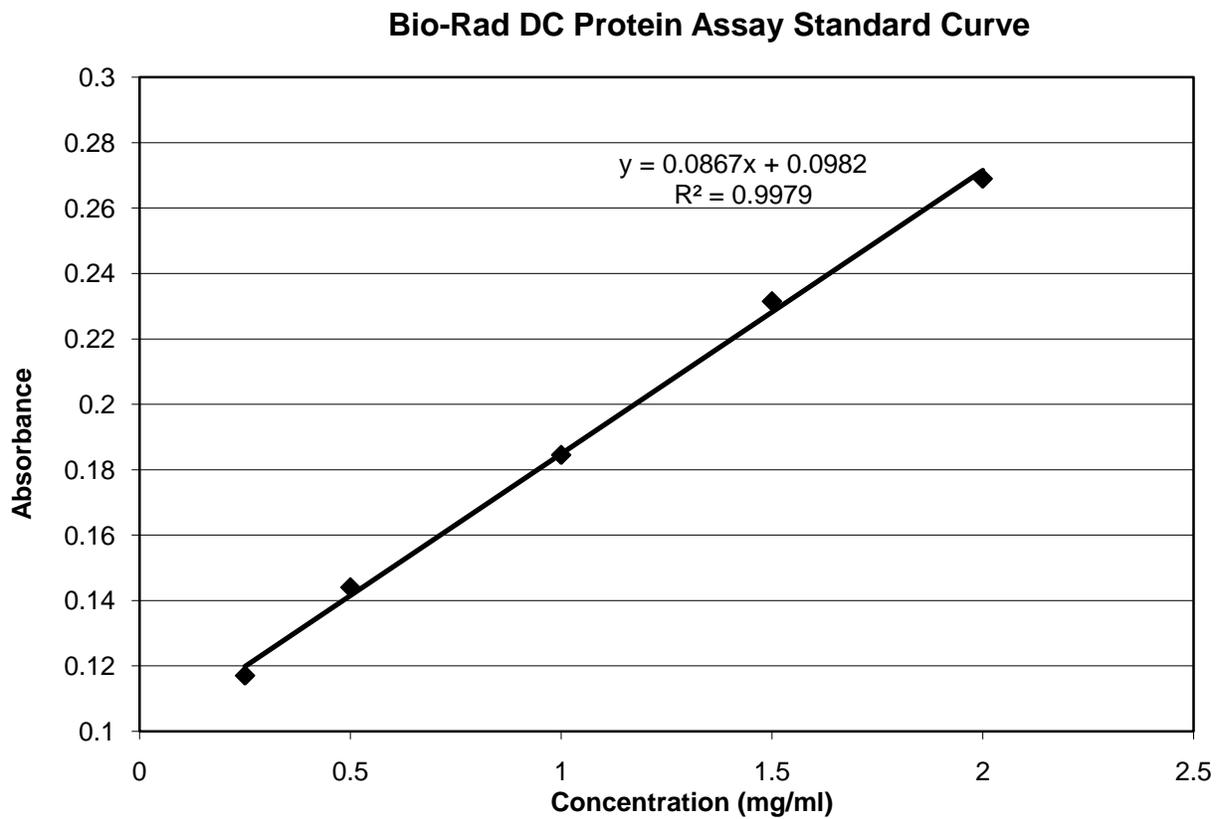Absorbance

Concentration (mg/ml)

Figure 4 shows a protein standard curve using the Bio-Rad DC Protein Assay. The absorbances were read at 750

nm with standards from 0.125 to 2.0 mg/ml.

Statistics/Data Analysis

Body weight (BW) changes throughout the 12 wk interventions were analyzed using a 1-way analysis of variance (ANOVA) with repeated measures; post-hoc Tukey HSD comparisons were used to determine individual group differences when a significant F ratio was obtained. Differences in initial body weight, final body weight, food intake, and hypoxyprobe concentration were determined using 1-way ANOVA with post hoc Tukey comparisons where appropriate. All analyses were done using SPSS version 16.0 (Chicago, IL) and data presented as mean ± SEM. Alpha level for main effects will be set at $p \leq 0.05$.

CHAPTER 4: RESULTS

Exercise and low fat diet attenuate body weight and epididymal adipose tissue gain

Following six weeks of ad libitum high fat diet treatment, diet-induced obese mice had significantly higher body weights than those fed a chow diet ($p<.001$). At four, eight, and the final 12 week time point, all groups were significantly different ($p<.05$) than all other groups with the exception of the low fat sedentary and low fat exercise groups, which did not differ significantly at any time point. Additionally, the high fat exercise and low fat sedentary groups did not differ significantly at the eight week time point only ($p=.135$). There was a significant time effect ($p<.001$) with all groups gaining weight over time. Significant time x diet ($p<.001$) and time x exercise ($p<.001$) interactions were seen, but there was no time x diet x exercise interaction. Pre and post intervention weights can be found in **table 2** and the time course of body weight gain can be seen graphically in **figure 5**.

Epididymal fat pad weights were also reduced by exercise and dietary interventions. All treatment groups were significantly different than other groups ($p<.05$) with the exception of the high fat sedentary and high fat exercise groups. There was both a significant exercise ($p=.01$) and diet effect ($p<.001$) but no combined exercise x diet interaction ($p=.128$). Mean values for epididymal fat pad weight can be found in **table 2** and can be seen graphically in **figure 6.** These results for body and fat pad weight are similar to those from a recently published study from our lab where mice underwent the same intervention (52).

Treatment	n	Initial Body Weight (g)	Final Body Weight (g)	Epididymal Fat Weight (g)
CHOW	14	$22.78 \pm .31^{*}$	$28.36 \pm .27^{b}$	$.665 \pm .03^{c}$
HFEX	15	$26.39 \pm .71$	39.33 ± 1.49^{d}	$2.315 \pm .12^{a}$
LFEX	15	$27.09 \pm .66$	$32.57 \pm .77^{a}$	$1.15 \pm .08^{d}$
HFSED	15	$27.35 \pm .61$	$44.33 \pm .82^{c}$	$2.39 \pm .13^{a}$
LFSED	14	$26.35 \pm .49$	$34.56 \pm .85^{a}$	$1.62 \pm .12^{b}$

Table 2 shows the body weights of mice before and after dietary and/or exercise interventions as well as epididymal fat pad weight as measured at sacrifice. *Signifies significant differences from all other groups and different letters correspond to differences between groups with significance set at p<.05.
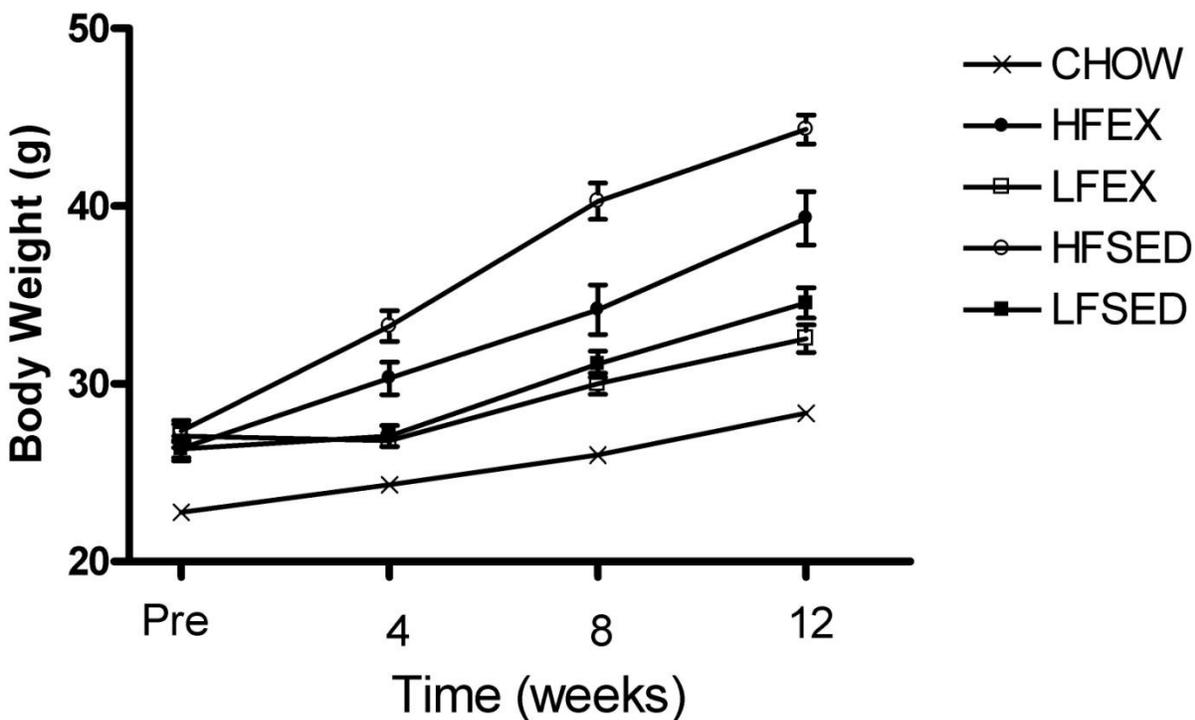

Figure 5 shows the body weights of the different treatment groups before intervention and at 4, 8, and 12 weeks.

Epididymal Fat Pad Weight
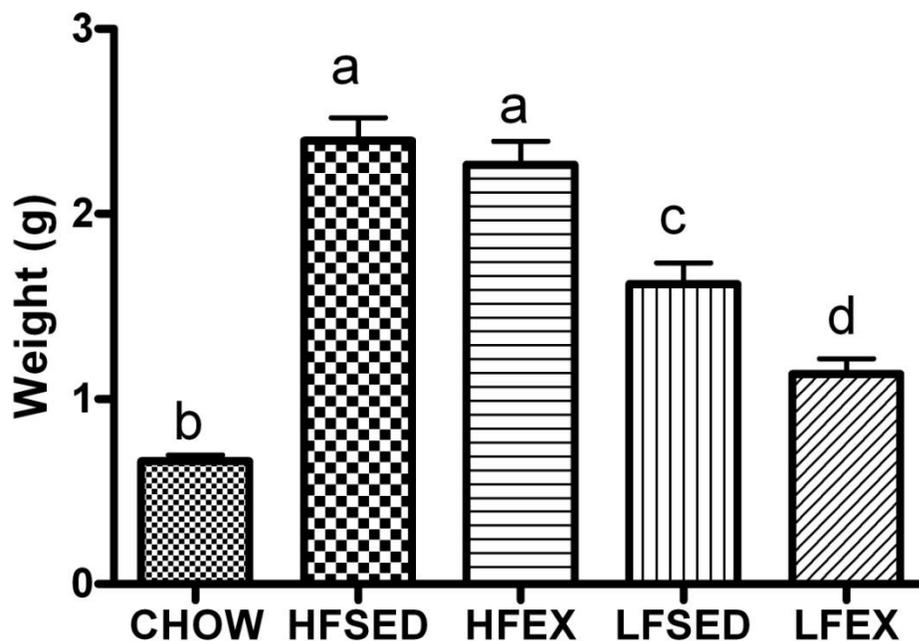## Following Treatment

Figure 6 shows epididymal fat pad weight following sacrifice. Only the high fat exercise and high fat sedentary group did not differ significantly in their final fat pad weight. Different letters correspond to significant differences between groups.

Exercise and low fat diet reduce adipose tissue hypoxia in obese mice

Following treatment, both low fat diet and exercise had an effect on reducing hypoxia (lower levels of hypoxyprobe) in the subset of mice injected with pimonidazole hydrochloride with the combined intervention (exercise and low fat diet) being significantly lower than all other groups. After log transformation to correct for abnormal data distribution, two way ANOVA's revealed a significant effect of both exercise ($F_{1,23}$=5.1; p=0.04) and diet ($F_{1,23}$=9.4; p=0.006) in reducing adipose tissue hypoxia with a tendency for low fat diet alone to be more efficacious than exercise alone. There was a significant exercise x diet interaction ($F_{1,23}$=6.3; p=0.02) such

that adipose tissue hypoxia in the combined intervention (low fat exercise) was significantly less than all other groups. Results for adipose tissue hypoxia as measured by hypoxyprobe ELISA can be seen in **figure 7**.

Effect of Exercise and Diet on Hypoxyprobe Concentration in Epididymal Fat
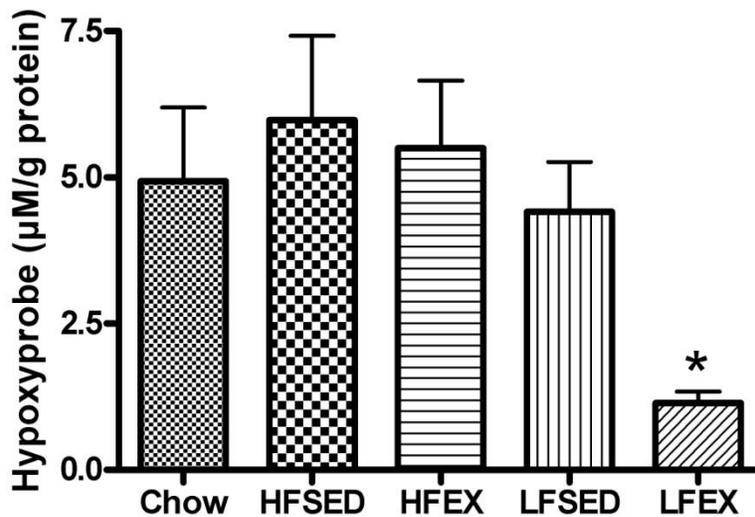
Figure 7 shows the effect of exercise and diet on hypoxia in obese mice as measured by Hypoxyprobe. Both exercise and low fat diet had independent and combined effects on reducing hypoxia in the epididymal adipose tissue with the combined intervention being significantly lower than all other groups.

CHAPTER 5: DISCUSSION

Findings

The most important finding of this study was that exercise training, in combination with a low fat diet, can reduce adipose tissue hypoxia. It has been well established that high fat fed mice exhibit increased levels of adipose tissue hypoxia (61,62,63), but it has not been shown that treatment, either by diet or physical activity intervention, can reduce or attenuate this effect. Data from this study suggest that both exercise and low fat diet play a role in reducing adipose tissue hypoxia in diet-induced obese mice. Interestingly, low fat diet had a greater effect than exercise with the combined intervention (low fat diet and exercise) having the most robust effect. This suggests that while both exercise and low fat diet likely play important roles in reducing adipose tissue hypoxia, the underlying physiological mechanisms causing these effects may be different with diet having the main effect and exercise only having an effect when combined with a low fat diet.

Recently published work from our lab (52) shows both exercise, low fat diet, and their combination results in significant reductions of obesity-induced increases in systemic and white adipose tissue inflammation. In this instance, the combination of moderate exercise with a reduction in caloric intake was more robust than either single intervention with exercise having unique anti-inflammatory effects. Similar results have been found using voluntary wheel running exercise (64). These results on white adipose tissue inflammation are similar to the present findings on adipose tissue hypoxia, suggesting that adipose tissue hypoxia may be a mechanism by which exercise and/or low fat diet reduces inflammation in obese mice, under this paradigm.

Limitations

There were some limitations to this study. The mode of exercise used in this study was treadmill running. Other studies have investigated changes in inflammation using voluntary wheel running as the preferred exercise mode (64). It is our belief that treadmill running is a more preferable mode of exercise in mice as the intensity and duration of the running can be strictly controlled. Under voluntary wheel running model, mice will often run greater than ten kilometers per day, representing an extreme amount of physical activity. Further, there is a large variability in distance run between mice and is impossible to control. We feel that treadmill training is more applicable to public health recommendations for physical activity and that it is the more appropriate model to examine the effect of exercise on mice.

Another potential limitation of the study was the use of hypoxyprobe (pimonidazole hydrochloride) as a marker of hypoxia in adipose tissue. Indeed, this is an indirect marker of hypoxia in adipose tissue and direct measurement of tissue partial pressure of oxygen (PO_2) is possible via an oxygen meter inserted directly into the tissue. This method was recently critiqued in a review by Ye (62). The PO_2 assay is able to provide definitive information about the about the level of oxygen in the tissue, but the assay requires special equipment and involves surgical operation. Furthermore, the cost is high as the oxygen probe is fragile and needs to be replaced frequently. Additionally, the assay is dependent on the sensitivity of the oxygen meter and skill of the investigator.

The primary advantage of the hypoxyprobe method is that the assay does not require special equipment and can be performed in most laboratories. This technique has been scrutinized for its inability to give a precise number regarding tissue oxygen pressure (62). However, this critique is in regard to western blot and histochemical staining. We believe that

by using ELISA to stain for hypoxyprobe, we are better able to quantify pimonidazole hydrochloride concentration within the tissue. Hypoxyprobe has been used extensively in research as a marker of hypoxia in adipose tissue (61,62,63) as well as a number of other tissues including brain, tumors, wounds, liver, heart, kidney, and others.

Future Directions

Though these findings suggest reduced/attenuated adipose tissue hypoxia as a potential mechanism by which exercise and low fat diet treatment may be reducing adipose tissue inflammation and the metabolic complication that follow in the obese state, some questions regarding this phenomenon remain unanswered. Indeed, hypoxia level alone does not paint a complete picture of what is going on in the adipose tissue in response to these hypoxic conditions.

Of particular interest is the response of HIF-1α levels in the adipose tissue to both exercise and low fat diet. As mentioned, HIF-1α is the master signal mediator of the cellular response to hypoxia and acts on a number of target genes including VEGF. This leads to the stimulation of angiogenesis in adipose tissue and generally results in excessive adipose tissue growth and development with obesity being the eventual outcome. We have shown previously that both exercise and low fat diet reduce leptin gene expression in adipose tissue (52). This is relevant as leptin is also a target gene for HIF-1 (50). Whether or not the reduced hypoxia seen in this study leads to changes in HIF-1α and VEGF-induced changes in angiogenesis is a critical question that should be addressed in future studies.

It should also be noted that a number of factors other than hypoxia have been cited as potential causes for adipose tissue inflammation and these factors should not be ruled out as

potential mechanisms by which exercise and low fat diet may be exerting their anti-inflammatory effect. These factors are likely related to the overall well-being of the adipose tissue and may include increased blood flow, reduced adipocyte size, reduced cellular stress, and improvements in mitochondrial function and fatty acid oxidation. Further research is needed on the effect of exercise and/or low fat diet treatment on these factors.

Future research should also investigate the mechanisms by which exercise and a low fat diet are reducing adipose tissue hypoxia. As mentioned, a number of possible causes have been proposed for the increase in adipose tissue hypoxia seen in obesity (62). These include reduced adipose tissue blood flow and, reduced capillary and endothelial density, and increased adipocyte size beyond the diffusion distance of oxygen. Future research should investigate these variables as potential mechanisms by which exercise and/or a low fat diet may be decreasing hypoxia in adipose tissue.

CHAPTER 6: SUMMARY AND CONCLUSION

Due to the increased prevalence and numerous health risks that accompany obesity, understanding its causes and underlying physiological mechanisms continues to be a high priority in current healthcare research. The rising rates of obesity are a result of increased caloric intake, particularly from a diet high in saturated fat, and reduced caloric expenditure through decreased levels of physical activity. Obesity is now being increasingly considered a state of chronic inflammation. Further, local adipose tissue inflammation, particularly in the white adipose tissue associated with central obesity, has been shown to be a cause of many of the chronic diseases and metabolic disturbances associated with obesity, including insulin resistance and cardiovascular disease. Adipose tissue is now being viewed as an active endocrine organ, releasing hormone-like cytokines that affect immune function and inflammatory processes throughout the body.

It has been well established that exercise can play a role in reducing systemic inflammation and recent work from our lab has shown that exercise can reduce inflammation locally in the adipose tissue. This reduced local inflammation contributes to improvements in systemic inflammation and the metabolic complications related to obesity. Given the significant role that exercise has in reducing adipose tissue inflammation and the association of adipose tissue inflammation with dysregulated metabolic function, exploring the mechanism by which exercise may reduce adipose tissue inflammation is imperative.

Adipose tissue hypoxia has recently been identified as a possible mechanism behind adipose tissue inflammation and its associated consequences. Indeed, adipose tissue hypoxia is related to increased levels of a number of inflammatory cytokines, likely acting through the

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activation of both HIF-1-α and NF-kB. Given this, investigating whether this phenomenon is a mechanism by which exercise decreases adipose tissue inflammation seemed like the logical next step.

Data from this experiment suggest that the adipose tissue hypoxia seen with obesity can be reduced through exercise when combined with a low fat diet, particularly when combined with a low fat diet. Of particular interest are the differential and combined effects of exercise and low fat diet on reducing or attenuating adipose tissue hypoxia. This suggests that exercise and physical activity interventions may act through different mechanisms to reduce hypoxia in the adipose tissue. These results are novel, being the first to show that adipose tissue hypoxia is reduced via exercise training. Further, this study is the first to show that the increased adipose tissue hypoxia seen in obesity can be slowed by treatment, either by exercise and/or low fat diet.

These results are very comparable to the reductions in inflammatory cytokines, including TNF-α, MCP-1, and F4/80, that were seen in recently published work from our lab (52) where diet-induced obese mice underwent the same treatment interventions as in this study. This connection makes it plausible to conclude that reduced adipose tissue hypoxia is a likely mechanism by which both exercise, low fat diet, and their combination are exerting their anti-inflammatory effects on adipose tissue.

Further studies investigating the mechanisms by which exercise reduces adipose tissue hypoxia are warranted. Likely causes include changes in adipocyte size, adipose tissue blood flow, and capillary and endothelial density and future studies should address these factors. Additional research is also needed on the series of molecular events initiated in response to hypoxia with HIF-1-α and VEGF being likely changed following hypoxia.

In conclusion, both exercise and low fat diet serve as effective treatments in reducing adipose tissue hypoxia in the obese state. This is likely serving as a mechanism to reduce adipose tissue inflammation. More research is needed to further understand this process. Additionally, exercise and low fat diet continue to act as effective treatment options for obesity.

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