

EFFECT OF HIGH-INTENSITY INTERVAL TRAINING ON INFLAMMATION

HIGH-INTENSITY INTERVAL TRAINING IMPROVES INSULIN SENSITIVITY
INDEPENDENT OF ADIPOSE TISSUE INFLAMMATION

By

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TITLE: High-Intensity Interval Training Improves Insulin Sensitivity Independent
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ABSTRACT

Obesity is associated with a state of chronic, low-grade inflammation that contributes to the development of insulin resistance and type 2 diabetes. The primary source of systemic, low-grade inflammation in obesity is macrophages that reside within adipose tissue. What causes adipose tissue macrophages to become inflamed in obesity is not fully understood, but increases in adipose tissue cell size, free fatty acids, and lipopolysaccharide have all been suggested to be important. The AMP-activated protein kinase (AMPK) is an important regulator of metabolism and has been shown to suppress adipose tissue macrophage inflammation in obesity.

Exercise is known to improve insulin resistance, and emerging evidence suggests that it also reduces adipose tissue inflammation. However, to date the relationship between exercise and inflammation has not been separated from the confounding effects of weight loss. The objectives of this study were to 1) determine whether high-intensity interval training improves insulin sensitivity in obese mice independent of weight loss and 2) assess the effect of exercise on the relationship between adipose tissue inflammation and insulin sensitivity.

In preliminary studies we demonstrated that high-intensity interval training (HIT) on a treadmill in lean and obese C57BL/6 mice (100% of maximal running speed for 2 min followed by 2 min of rest over 60 min) increased the phosphorylation of AMPK in adipose tissue. To assess the chronic effects of exercise training in obesity, C57BL/6 mice were assigned to one of three groups: a control, low-fat, chow diet (Chow), 12

weeks of high-fat diet with no exercise (HFD Sed), or 6 weeks of high-fat feeding followed by an additional 6 weeks of HIT 3 times per week.

Consumption of a high-fat diet increased body mass and adiposity compared to chow-fed mice. Similarly, measures of insulin resistance and adipose tissue macrophage infiltration and inflammation were increased with high-fat feeding. HIT had no effect on body mass, epididymal fat mass, adiposity, or adipose tissue cell size. HIT also did not alter adipose tissue inflammation, macrophage infiltration, or adipose tissue macrophage polarization/inflammation. Nevertheless, when compared to HFD Sed mice, HIT resulted in lower fasting insulin levels and improved glucose tolerance and insulin sensitivity, as measured by intraperitoneal glucose and insulin tolerance tests, respectively. Surprisingly, conscious hyperinsulinemic-euglycemic clamps revealed that improvements in whole-body insulin sensitivity following HIT were not the result of improved skeletal muscle glucose uptake, but instead appeared to be primarily the result of enhanced insulin sensitivity in liver and adipose tissue.

In conclusion, these findings demonstrate that HIT improves whole-body insulin sensitivity and glucose homeostasis independent of changes in body mass or adipose tissue inflammation. The benefits of exercise in obese individuals are obvious; however, the mechanisms underlying the improvements in insulin sensitivity observed following chronic HIT remain to be elucidated.

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LIST OF ABBREVIATIONS

2-[¹⁴ C]-DG	2-deoxy-D-[¹⁴ C]glucose
ABC	Avidin-biotin enzyme complex
AMPK	AMP-activated protein kinase
AUC	Area under the curve
Ba(OH) ₂	Barium hydroxide
BMI	Body mass index
CLAMS	Comprehensive lab animal monitoring system
CLS	Crown-like structure
CRP	C-reactive protein
CT	Computed tomography
DAB	Diaminobenzidine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FFA	Free fatty acid
GTT	Glucose tolerance test
H&E	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High-fat diet

HIT	High-intensity interval training
HOMA-IR	Homeostatic model of assessment-insulin resistance
ICAM1	Interceullular adhesion molecule 1
IKK β	Inhibitor of nuclear factor κ B kinase β
IL1 β	Interleukin-1 β
IL1ra	Interleukin-1 receptor antagonist
IL6	Interleukin-6
IL10	Interleukin-10
iNOS	Inducible nitric oxide synthase
IRS1	Insulin receptor substrate 1
JAK	Janus kinase
JNK	c-Jun kinase
LPS	Lipopolysaccharide
MCP1	Monocyte chemotactic protein 1
NaCl	Sodium chloride
NaF	Sodium fluoride
NaVO ₄	Sodium orthovanadate
NF- κ B	Nuclear factor κ B
PAI1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
RER	Respiratory exchange ratio
SEM	Standard error of the mean

sFA	Saturated fatty acid
SOCS3	Suppressor of cytokine signaling 3
STAT	Signal transducer and activator of transcription
TLR4	Toll-like receptor 4
TNF α	Tumor necrosis factor α
VCO ₂	Volume of carbon dioxide
VO ₂	Volume of oxygen
WHO	World Health Organization
ZnSO ₄	Zinc sulphate

1. INTRODUCTION

1.1 Obesity and the Metabolic Syndrome

The prevalence of obesity has become an epidemic, affecting more than 500 million people worldwide (WHO). Canada is not exempt: 23% of Canadian adults are obese and an additional 36% are overweight (Statistics Canada). The statistics are alarming; however, the rapidity of the increase in rates of obesity is especially concerning. Since 1972, the prevalence of obesity in Canada has more than doubled. As obesity has become an epidemic, co-morbidities associated with obesity have also increased, including insulin resistance, dyslipidemia, abdominal obesity, hypertension, high blood pressure, and hyperglycemia. Together, these factors comprise the metabolic syndrome, which is a risk factor for the development of type 2 diabetes, cardiovascular disease, fatty liver disease, and certain cancers (1-3).

As a result, significant medical and economic burden has been associated with obesity. In fact, a recent meta-analysis reported that the cost of healthcare for obese individuals is 30% greater than that of their normal weight peers (4). The strains on personal health and quality of life, as well as corporate and individual financial interests, have moved the investigation into the causes and treatments of obesity to the forefront of research efforts.

1.2 Obesity and Inflammation

1.2.1 Obesity as a State of Chronic, Low-Grade Inflammation

An interesting observation about obesity is the presentation of chronic, low-grade inflammation, particularly in the adipose tissue. The first evidence of an association between obesity and inflammation was established by Hotamisligil *et al.*, who determined that the pro-inflammatory cytokine tumor necrosis factor α (TNF α) was expressed at higher levels in the adipocytes of obese rodents compared to lean rodents (5). Since Hotamisligil's original study, researchers have found additional links between adiposity and the expression of a variety of inflammatory cytokines, including interleukin-1 β (IL1 β) (6-8), interleukin-1 receptor antagonist (IL1ra) (7, 9, 10), interleukin-6 (IL6) (6, 9, 11-20), interleukin-8 (IL8) (16, 20-22), interleukin-10 (IL10) (12, 21), and interleukin-18 (IL18) (12, 23).

Although the adipose tissue is known to be an endocrine organ that secretes adipocyte-derived cytokines, the primary source of inflammatory molecules released in obesity appears to be macrophages resident in the adipose tissue (24). During obesity, the increase in body mass index (BMI) is directly related to the accumulation of macrophages in the adipose tissue (24, 25). In fact, as the proportion of macrophages within the adipose tissue increases so too does the expression of pro-inflammatory cytokines (26).

1.2.2 Why Do Macrophages Infiltrate Adipose Tissue in Obesity?

Although macrophage infiltration into adipose tissue during obesity has been firmly established, the mechanisms driving recruitment remain unknown. Some

hypotheses include: lipid oversupply, hypoxia and adipocyte cell death, and increased expression of cytokines that initiate recruitment (Figure 1.1).

In many cases, the development of obesity is associated with over-nutrition, including lipid oversupply. Saturated fatty acids, in particular, have been linked with instigating inflammation via the activation of macrophage toll-like receptor 4 (TLR4) (27-30). Upon stimulation, TLR4 activates the IKK β /NF- κ B signaling cascade, ultimately resulting in translocation of NF- κ B into the nucleus to upregulate the transcription of inflammatory cytokines (31, 32).

As the adipose tissue expands in obesity, adequate vascularization often fails to occur (33, 34), creating a hypoxic environment (35, 36). Not only does hypoxia directly increase the expression of inflammatory genes in both adipocytes and adipose tissue macrophages, but it also results in adipocyte death (35). Consequently, macrophages infiltrate the adipose tissue to phagocytose the dead or dying cells.

Finally, adipose tissue expansion increases the expression of signaling molecules that promote macrophage infiltration, including monocyte chemoattractant protein 1 (MCP1), adhesion molecules, and leptin. The expression of MCP-1, a chemo-attractant for macrophages, increases with adipose tissue hypertrophy, effectively recruiting circulating bone-marrow derived monocytes into the adipose tissue (24, 37). Endothelial cell adhesion molecules are necessary for immune cells to migrate from the circulation into the adipose tissue. During obesity the expression of intercellular adhesion molecule 1 (ICAM1) increases, which facilitates the transport of macrophages into the adipose tissue (38, 39). Lastly, leptin, a well-studied protein hormone associated with metabolism and

appetite, may also attract macrophages to the adipose tissue, either by directly recruiting immune cells (40, 41) or by increasing the expression of endothelial cell adhesion molecules (42).

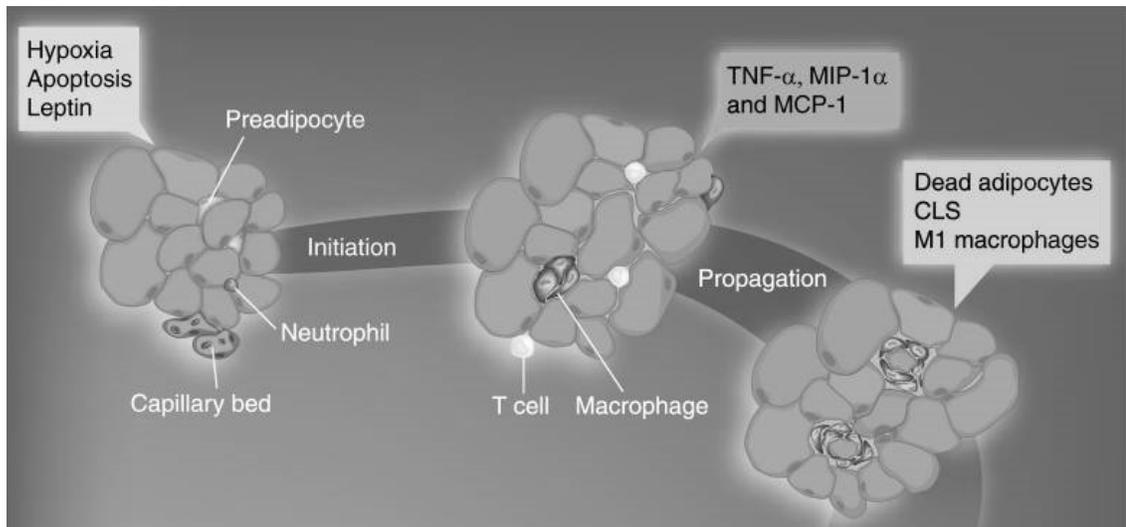


Figure 1.1 Macrophage Infiltration into Adipose Tissue in Obesity Stages of initiation and propagation of macrophage infiltration into adipose tissue during obesity. Factors leading to macrophage infiltration include hypoxia, cell death, and expression of chemotactic cytokines, such as leptin. The inflammatory state is propagated by increased expression of inflammatory cytokines, primarily by resident macrophages, ultimately leading to adipocyte death, the formation of crown-like structures, and a shift in macrophage polarization towards the M1 phenotype. TNF α : tumor necrosis factor α ; MIP1 α : macrophage inflammatory protein 1 α ; MCP1: monocyte chemotactic protein 1; CLS: crown-like structure. **Adapted from Surmi and Hasty, 2008.**

1.2.3 Characteristics of Adipose Tissue Macrophages in Obesity

During obesity, adipose tissue macrophages display unique characteristics that are not observed in lean individuals, including a shift in polarization towards a pro-inflammatory phenotype and the formation of crown-like structures. In fact, newly recruited macrophages have a higher capacity for inflammation compared to resident macrophages (26).

Adipose tissue macrophages can be identified by a variety of markers, including F4/80, CD68, and CD11b. F4/80, a glycoprotein found on the surface of most mouse macrophages, is the most extensively used macrophage-specific marker (43). Similarly, macrosialin, a CD68 homolog, is highly and specifically expressed by mouse macrophages (44). More specifically, F4/80(+)CD11c(+) macrophages are observed in the adipose tissue of obese, but not lean, mice (45). And, F4/80(+)CD11b(+)CD11c(+) cells from the adipose tissue appear to have heightened inflammatory properties in diet-induced obese mice compared to CD11c(-) cells (30).

While many different subpopulations of macrophages likely exist at any given time, macrophages are largely grouped as either classically or alternatively activated. Classically activated, or M1, macrophages typically express pro-inflammatory cytokines, while alternatively activated, or M2, macrophages express anti-inflammatory cytokines. Inflammatory markers that characterize M1 macrophages include TNF α , IL6, IL1 β , and the induction of iNOS. M2 macrophages are associated with increased production of the anti-inflammatory cytokine IL10 and arginase (46). In the adipose tissue of lean mice, macrophages are typically alternatively activated (M2). But, during obesity the polarization of adipose tissue macrophages changes from M2 to M1 (26). Interestingly, the change in polarization of adipose tissue macrophages may not be caused by a “switch” of resident M2 macrophages to the M1 polarization, but appears to be due to increased recruitment of inflammatory M1 macrophages from the circulation (46, 47). Regardless, the shift from M2 to M1 adipose tissue macrophages has significant implications for insulin resistance—M2 macrophages have the potential to protect the

adipose tissue from inflammation, while M1 macrophages have been shown to contribute to insulin resistance (26).

An important characteristic of inflammatory macrophages found in the adipose tissue is the formation of crown-like structures (CLS) (Figure 1.2). Large, multi-nucleate aggregations form around dead adipocytes as the macrophages scavenge free fatty acids (48). In obese mice, these macrophages display a unique dendritic-cell specific marker, CD11c+, which is not found on macrophages of lean mice and is likely involved in the formation of the CLS (46).

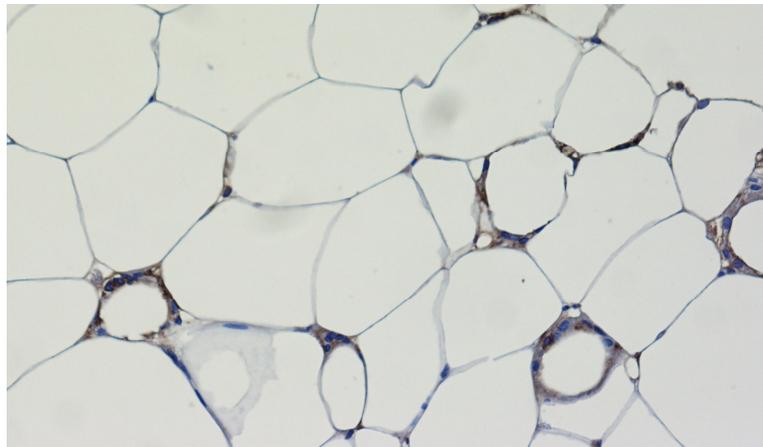


Figure 1.2 Crown-Like Structures Crown-like structures are characterized by multiple macrophages aggregating around a dead adipocyte. Brown colour represents F4/80+ cells. Image shown at 100X magnification.

1.2.4 Propagation of Inflammation

Adipose tissue macrophages not only contribute to the development of chronic, low-grade inflammation but also propagate the state of inflammation in obesity. As noted above, macrophages are the primary source of inflammatory cytokines in the adipose tissue. In addition to producing cytokines in response to the environment of over-

nutrition found in obesity, inflammatory cytokines released by neighboring macrophages also activate pathways that increase inflammatory cytokine expression. Further, adipose tissue macrophages secrete chemotactic cytokines, such as monocyte chemoattractant protein 1 (MCP1), that recruit additional macrophages to the adipose tissue and exacerbate the development of obesity-induced inflammation (25, 49).

1.3 Adipose Tissue Inflammation and Insulin Resistance

1.3.1 Inflammation and Insulin Signaling

As one of the most widely studied inflammatory cytokines, TNF α provides a valuable example of how pro-inflammatory cytokines directly affect insulin signaling and glucose uptake (50). In healthy individuals, insulin stimulates the insulin receptor to phosphorylate tyrosine residues of insulin receptor substrate 1 (IRS1), which is required for the normal biological action of insulin on a cell. However, TNF α interferes with the insulin receptor and decreases insulin-stimulated tyrosine phosphorylation, causing a blunted, or resistant, response to insulin (51).

In addition to directly interfering with the insulin signaling cascade, inflammatory cytokines activate pathways that are known to negatively affect insulin sensitivity. An important regulator of inflammatory cytokine expression that also affects insulin signaling is nuclear factor κ B (NF κ B). In response to inflammatory signals, I κ B kinase β (IKK β) is activated and phosphorylates I κ B, which results in the degradation of I κ B and liberation of NF κ B, allowing NF κ B to translocate into the nucleus (52). In the nucleus, NF κ B controls DNA transcription and has been specifically linked to regulating the

transcription of inflammatory cytokines. Interestingly, NF κ B binding in macrophages is significantly correlated with BMI. In obese individuals, NF κ B binds DNA more readily than in lean individuals, increasing the transcription of pro-inflammatory cytokines (31, 32).

Another mediator of inflammatory signaling, c-Jun kinase (JNK) is activated in metabolically active tissues, including the liver, muscle, and adipose tissue, by stress signals, whether in the form of pathogens, pro-inflammatory cytokines, or free fatty acids (FFA). JNK activity not only causes an increase in inflammatory cytokine production, but also phosphorylates and inhibits IRS1 at Ser307, which disrupts insulin signaling and contributes to insulin resistance (53-55). In confirmation of the role of JNK in mediating cross-talk between metabolic and immune systems, when JNK1 was deleted from hematopoietic cells, mice fed a high-fat diet were protected from developing chronic, low-grade inflammation and insulin resistance, despite becoming obese (55).

The suppressor of cytokine signaling 3 (SOCS3) also represents a link between metabolism and immunity. SOCS3 expression increases in response to the activation of the Janus kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathway by inflammatory cytokines (56-58). Subsequently, SOCS3 interferes with the insulin receptor and inhibits tyrosine phosphorylation of IRS1, preventing normal insulin signaling (59-61). In addition, in adipose tissue, SOCS3 also promotes the degradation of IRS1, which further contributes to a disruption in the insulin signaling pathway (62-64).

Toll-like receptor 4 (TLR4) appears to be an important mediator that allows for cross-talk between immune and metabolic pathways. Typically, toll-like receptors are

activated by pathogens and respond by mobilizing innate immune responses. Interest in TLR4 in the context of metabolic disorders has surfaced as a result of the receptor's activation by both lipopolysaccharide (LPS) and saturated fatty acids (sFA). When TLR4 is activated, the intracellular inflammatory pathways associated with insulin resistance, including the IKK β signaling cascade, are activated. In studies using a loss-of-function mutation in the gene encoding TLR4, mice were protected against the development of diet-induced obesity and insulin resistance. The reasons for the protection include decreased adiposity, lower levels of JNK and IKK β , and partial protection from diet-induced insulin resistance (29, 32).

1.3.2 Macrophages and Insulin Sensitivity

With the knowledge that inflammation interferes with insulin signaling and that macrophages are the primary source of inflammatory cytokines in the adipose tissue, focus has turned to investigate the role of inflamed adipose tissue macrophages in insulin resistance. As described above, IKK β and JNK represent important inflammatory pathways that contribute to insulin resistance. Indeed, disabling IKK β and JNK in macrophages protects mice from diet-induced insulin resistance (55, 65). In addition, depleting the CD11c⁺ cell population, which represents adipose tissue macrophages recruited to the adipose tissue in obesity, normalizes inflammatory status and improves insulin sensitivity in obese mice (66).

Recent work completed by the Steinberg laboratory and others indicates that macrophage AMP-activated protein kinase (AMPK) may be critical in suppressing the

inflammatory response in obesity (Galic *et al.*, in review, 52, 67-69). AMPK is a master metabolic regulator that maintains energy homeostasis and is activated in a variety of tissues with exercise. In addition, AMPK regulates inflammation in macrophages by suppressing pro-inflammatory responses and directing macrophage polarization towards an anti-inflammatory phenotype (52). In the adipose tissue of obese individuals, a decrease in AMPK activity is closely linked with an increase in inflammation (67). AMPK activity in macrophages is sensitive to changes in inflammation: Stimulation by anti-inflammatory cytokines results in AMPK phosphorylation, while treatment with LPS or FFA, which induce a pro-inflammatory response, cause the dephosphorylation of AMPK (52, 68). Finally, berberine, a plant-alkaloid based medication that improves symptoms of the metabolic syndrome, appears to act by repressing inflammation via AMPK (69).

In the Steinberg lab, lethally irradiating wild-type mice and transplanting bone marrow from AMPK β 1-knock out mice resulted in almost complete elimination of macrophage AMPK activity. On a high-fat diet, hematopoietic deletion of AMPK β 1 resulted in systemic and adipose tissue inflammation and insulin resistance (Galic *et al.*, in review). The identification of macrophage AMPK as a key regulator of adipose tissue macrophage inflammation represents a crucial cross-point for targeting insulin resistance from aspects of both immune and metabolic pathways.

1.4 Anti-Inflammatory Effects of Exercise

1.4.1 Anti-Inflammatory Effect of Exercise in Adipose Tissue

Beyond reducing body mass, exercise is a well-known treatment for a number of metabolic complications associated with obesity, including improvements in glucose tolerance, lipid profile, blood pressure, and risk for cardiovascular disease (70, 71). However, exercise also appears to cause a reduction in inflammation, both systemically and in the adipose tissue.

A variety of studies have utilized chronic, low-intensity exercise protocols to establish a relationship between exercise and reductions in adipose tissue inflammation. In chow-fed rats exercised for at least 8 weeks, the inflammatory profile of adipose tissue significantly improved, as evidenced by reduced pro-inflammatory cytokine expression and an improved IL10/TNF α ratio in white adipose tissue (72-74). Similarly, the expression of pro-inflammatory cytokines, both in serum and white adipose tissue, was lower in diet-induced obese mice that underwent either voluntary or low-intensity exercise in comparison to sedentary mice (75, 76). However, an important caveat of these studies is that in all cases improvements in adipose tissue inflammation were accompanied by reductions in body mass. Given that adiposity/adipocyte size is the strongest predictor of the percentage of macrophages in the adipose tissue (24), it is impossible to conclude from these studies whether the reduction in inflammation was related to exercise or merely the result of a negative energy balance inducing weight loss.

While low-intensity exercise may reduce adipose tissue inflammation, exhaustive exercise appears to have a pro-inflammatory effect in adipose tissue. Exhaustive exercise

in chow-fed rats, achieved by 50min of treadmill running at 70% VO_2 max followed by increasing intensity by 1m/min every 1 min until exhaustion, decreased the IL10/TNF α ratio in white adipose tissue and increased IL6 expression (77). Similarly, IL6 and IL10 expression was increased in the adipose tissue of over-trained, chow-fed rats, potentially via exacerbation of TLR4 and NF κ B pathways (78).

1.4.2 Activation of AMPK in Adipose Tissue with Exercise

A discussion about the effects of exercise would be incomplete without mention of AMPK. In the adipose tissue, both acute and chronic exercise activate AMPK in adipose tissue to regulate lipid metabolism, increasing fatty acid oxidation and decreasing esterification to increase the availability of fatty acids to be used as a fuel source for contracting muscle (79-81). Activation of AMPK with exercise is particularly induced in the visceral depot, as opposed to the subcutaneous adipose tissue (82). The activation of AMPK in adipose tissue may be due to local changes in energy status (83) or a response to circulating catecholamines that are increased with exercise (80, 84). Together with the novel realization that AMPK plays a critical role in mediating adipose tissue macrophage inflammation, AMPK may be responsible for the cross-talk between the effects of exercise and inflammation.

1.5 High-Intensity Interval Training

High-intensity interval training (HIT) involves short bouts of high-intensity exercise alternating with periods of rest. Typically, strength-training causes muscle

hypertrophy (85, 86), while endurance exercise increases mitochondrial biogenesis and oxidative capacity (87-89). However, high-intensity interval training maintains the metabolic adaptations typically associated with endurance training in combination with a training volume that is approximately ten times lower than traditional endurance exercise (90). After only 2 weeks of HIT, insulin sensitivity and glucose homeostasis significantly improve (91, 92). In addition, high-intensity interval training activates AMPK in skeletal muscle, which may be driving the increases in mitochondrial biogenesis and skeletal muscle oxidative capacity (93). As a result, utilizing high-intensity interval training will avoid the reductions in body mass typically associated with exercise studies and activate AMPK to enable a more thorough investigation into the effect of exercise on adipose tissue inflammation.

2. STUDY OBJECTIVES

2.1 Rationale

The relationship between exercise, adipose tissue inflammation, and insulin sensitivity remains unclear. While a variety of studies have investigated the role of exercise in adipose tissue inflammation, the effects of exercise independent of weight loss have not been elucidated. Knowing that body mass also plays the most important role in dictating adipose tissue inflammation, separating the effect of weight loss is critical to understanding how exercise affects inflammation. In addition, as new research points towards AMPK as an important mediator of adipose tissue inflammation, utilizing exercise protocols that maximally activate AMPK are crucial to investigating the full effect of exercise on inflammation. Previous studies have utilized low-intensity exercise protocols; however, AMPK activation increases with training intensity. Therefore, utilizing high-intensity interval training that has been shown to activate AMPK in adipose tissue will allow for a more thorough investigation into the effects of exercise on inflammation and insulin sensitivity.

2.2 Hypothesis

High-intensity interval training reduces adipose tissue inflammation independent of weight loss, contributing to improved insulin sensitivity in diet-induced obese mice.

2.3 Objectives

The objectives of the present study are to:

1. Determine whether high-intensity interval training increases the activity of AMPK in adipose tissue.
2. Determine whether chronic high-intensity interval training improves insulin sensitivity in diet-induced obese mice independent of weight loss.
3. Determine whether high-intensity interval training reduces adipose tissue inflammation in diet-induced obese mice independent of weight loss.

3. METHODS

3.1 Pilot Studies

To our knowledge, no studies to date have examined the effects of high-intensity interval training (HIT) on inflammation and insulin sensitivity in mice. Therefore, we conducted a number of pilot studies to ensure that HIT activates AMPK in the adipose tissue, both in chow-fed and high-fat diet-fed mice, and improves glucose tolerance in obese mice.

First, a pilot study was conducted to assess whether an acute bout of HIT would activate AMPK to a greater degree than endurance exercise. Chow-fed mice were either exercised for 60 minutes at 15m/min (endurance exercise) or 60 minutes alternating between 30m/min for 2 minutes and 2 minutes of rest (HIT). Immediately following the exercise bout, mice were sacrificed and tissues were collected and snap frozen. Subsequently, tissues were homogenized, and the protein expression of AMPK T172 phosphorylation was assessed by western blotting.

Second, a pilot study was conducted in high-fat diet-fed mice to assess the effect of chronic HIT on AMPK activation and glucose tolerance in diet-induced, obese mice. Mice were fed a high-fat diet (45% kcal fat, D12451, Research Diets; New Brunswick, NJ) for 10 weeks before beginning high-intensity interval training. For the subsequent 5 weeks, mice were exercised for 60min, 3 days/week using cycles of 2min of high-intensity treadmill running and 2min of rest. Glucose tolerance tests were conducted before and after the exercise intervention. At the conclusion of the exercise intervention,

mice were sacrificed and tissues were collected and snap frozen. AMPK T172 phosphorylation, particularly in adipose tissue, was assessed by western blotting.

3.2 Study Design

Experimental protocols were approved by the McMaster University Research Ethics Board (AUP#09-05-15). Male, C57BL/6 mice were housed in SPF-level cages in the Central Animal Facility and maintained on a 12 hour light/dark cycle. Starting at 6 weeks of age (designated Week 0), mice either received a high-fat diet (45% kcal fat, D12451, Research Diets; New Brunswick, NJ) for 12 weeks or remained on a normal chow diet (17% kcal fat, Teklad 22/5, Harlan Laboratories; Indianapolis, IN) (Chow) with *ad libitum* access to food and water (Table 3.1).

Table 3.1 Composition of Chow and High-Fat Diets

	Chow Diet		High-Fat Diet	
	%g	%kcal	%g	%kcal
Protein	22	29	24	20
Carbohydrate	40.6	54	41	35
Fat	5.5	17	24	45
kcal/g	3.0		4.73	

After 6 weeks, high-fat diet-fed mice were assigned to either sedentary (HFD Sed) or exercise (HFD Ex) groups for an additional 6 weeks. Body weights of the mice were monitored weekly. Fed and fasting blood samples, glucose and insulin tolerance tests, CT scans, substrate utilization analyses, and hyperinsulinemic-euglycemic clamps were conducted in each group according to a common timeline, of which further details are provided in the sections below (Figure 3.1).

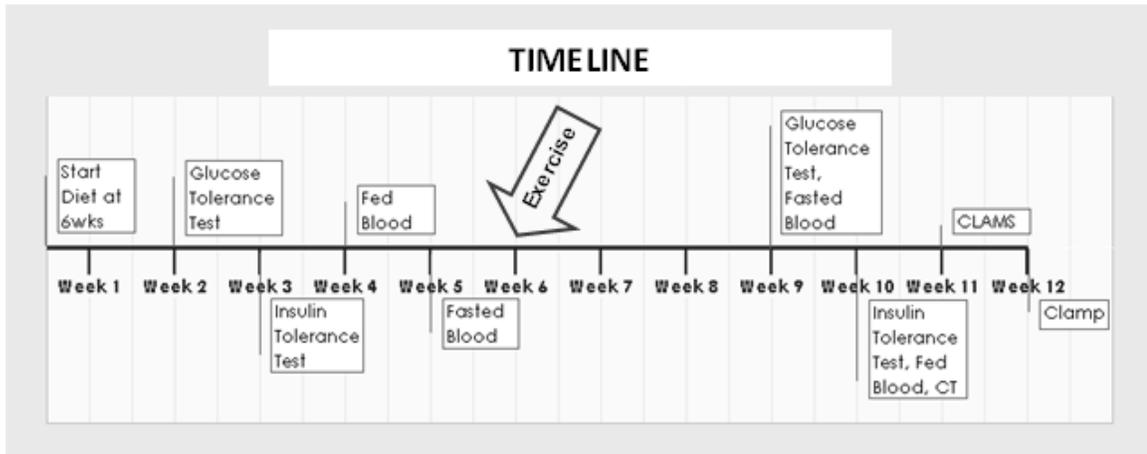


Figure 3.1 Timeline

3.3 Exercise Capacity Tests

Exercise capacity tests were conducted utilizing the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH). After equilibrating and calibrating the system, mice were placed in an enclosed treadmill to measure VO_2 and RER over the course of the test. Beginning at 10m/min and increasing by 1m/min every 2 minutes, mice ran until exhaustion, and the maximum speed and time to exhaustion were recorded.

3.4 Exercise Protocol

Following 6 weeks of high-fat feeding, mice assigned to the HFD Ex group were exercised using a high-intensity interval training protocol. During the preceding week, mice had been acclimatized to the treadmill over 3 days, running at 10-15 m/min for 15 minutes. High-intensity interval training involved cycles of 2min of high-intensity treadmill running followed by 2min of rest for 60min. Mice were exercised 3 days/week

for 6 weeks, with the speed increasing from 15m/min to 22 m/min to maintain a high-intensity workload at 100% of maximal running speed.

3.5 Serum Glucose, Insulin, and Adipokine Analyses

Fed and fasting blood samples were collected to analyze blood glucose and serum insulin and adipokine levels. In HFD Ex mice, blood samples were collected 24 hours after exercise to avoid an acute exercise effect. Mice were anaesthetized using isofluorane (Pharmaceutical Partners of Canada Inc.; Richmond Hill, ON), and blood was collected from the facial vein. Fasting samples were collected following an over-night, 12 hour fast. Blood glucose was measured using the Accu-Check Aviva blood glucose meter (Roche Diagnostics; Mannheim, Germany). After allowing blood to clot for approximately 30 minutes, whole blood was spun at 7500rpm for 7 minutes, and serum was collected and stored at -80°C. Serum insulin was measured using the Rat/Mouse Insulin ELISA kit from Millipore (St. Charles, MO) according to manufacturer's instructions. In addition, homeostasis model of assessment – insulin resistance (HOMA-IR) was calculated using fasting glucose and insulin values with the following formula: $HOMA-IR = \text{fasting glucose (mmol/L)} \times \text{fasting insulin (mU/L)} / 22.5$. Serum adipokines, including IL6, leptin, PAI1, and resistin were measured using the Mouse Serum Adipokine kit from Millipore (St. Charles, MO) according to manufacturer's instructions.

3.6 Glucose and Insulin Tolerance Tests

Glucose tolerance tests were performed twice over the course of the study to assess the animals' capacity to respond to a glucose load. Following a 6 hour fast, mice were weighed and received 1g/kg glucose delivered intraperitoneally. Blood glucose was measured from the tail vein at baseline and 20, 40, 60, 90, and 120 minutes after the glucose injection using the Accu-Chek Aviva blood glucose meter (Roche Diagnostics).

Similarly, insulin tolerance tests were conducted twice during the study to assess the animals' response to exogenous insulin. Again, mice were fasted for 6 hours, and 1U/kg of insulin was delivered intraperitoneally. Blood glucose was measured in the same way as the glucose tolerance test. Data were expressed as a percent of the baseline blood glucose and area under the curve was calculated using GraphPad Prism 5 (GraphPad Software Inc.; La Jolla, CA).

3.7 Food Intake and Energy Expenditure

The Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH) was used to assess RER, substrate utilization, food intake, and activity levels. After equilibrating and calibrating the system and allowing animals to acclimatize to their surroundings for approximately 12 hours, volume of oxygen consumed (VO_2), volume of carbon dioxide produced (VCO_2), food intake, and activity measurements were collected over the subsequent 72 hours. RER, carbohydrate oxidation, and lipid oxidation were calculated using the following formulas: $RER =$

VCO_2/VO_2 ; carbohydrate oxidation (kcal/hr) = $((4.585*VCO_2)-(3.226*VO_2)*4)/1000$;

lipid oxidation (kcal/hr) = $((1.695*VCO_2)-(1.701*VO_2)*9)/1000$.

3.8 CT Scans

Computed tomography (CT) scans were taken of the mice to assess body composition, specifically total adiposity. CT scans were conducted at the Animal Imaging Facility at McMaster University under inhalant anesthetic (isoflurane, Pharmaceutical Partners of Canada Inc.; Richmond Hill, ON). 1024 projections (1184x1120 pixels) were obtained over 360° using a GammaMedica Ideas X-SPECT system (Northridge, CA) and reconstructed to 512x512x512 voxel images using a modified Feldkamp algorithm (EXXIM; Pleasanton, CA). Images were analyzed using Amira 5.2.2 (San Diego, CA) to select only the adipose tissue based on the density of the image (Appendix I).

3.9 Hyperinsulinemic-Euglycemic Clamp

The hyperinsulinemic-euglycemic clamp was conducted at the conclusion of the study. Three days before the clamp procedure, a 25 gauge catheter was surgically inserted into the right jugular vein. Prior to the clamp, mice were fasted for 6 hours and placed in restrainers. After measuring the basal blood glucose level, D-[³H]-glucose was infused at a rate of 7.5µCi/hr for 1 hour to determine basal glucose disposal. Following collection of a basal blood sample from the tail vein, mice received a continuous infusion of insulin (10mU/kg/min) containing 7.5µCi/hr D-[³H]-glucose. As blood glucose

dropped, a 50% dextrose solution was infused and titrated to achieve euglycemia. After euglycemia was maintained for 30 minutes, a clamped blood sample was collected from the tail vein. Specific activity was measured in blood samples following lysis and deproteinization with $\text{Ba}(\text{OH})_2$ and ZnSO_4 . Basal and clamped glucose disposal rate and hepatic glucose output were calculated using Steele's equation for steady state conditions.

3.10 Tissue-Specific Glucose Uptake

To measure tissue-specific glucose uptake following the hyperinsulinemic-euglycemic clamp, a 100 μL bolus of 2-deoxy-D- ^{14}C -glucose (2- ^{14}C -DG) was infused into the catheter after collection of the clamped blood glucose sample. As a non-metabolizable glucose analogue, 2- ^{14}C -DG is phosphorylated to 2- ^{14}C -DG-6-P and remains in the target tissues following uptake. Therefore, the accumulation of 2- ^{14}C -DG-6-P enables the analysis of insulin-stimulated glucose uptake to specific tissues.

Blood samples were collected 10, 20, and 30 minutes following 2- ^{14}C -DG infusion, and specific activity was measured following lysis and deproteinization with $\text{Ba}(\text{OH})_2$ and ZnSO_4 . Following collection of the 30 minute blood sample, mice were anaesthetized using ketamine-xylazine (ketamine, Bimeda-MTC Animal Health Inc.; Cambridge, ON; xylazine, Bayer Health Care; Toronto, ON), and tissues were collected and snap frozen in liquid nitrogen. In addition, samples of white adipose tissue were preserved in formalin for histological analysis and serum was collected and stored at -80°C .

Subsequently, approximately 30mg of gastrocnemius, 30mg of liver, and 100mg of adipose tissue were chipped from whole tissues and weights were recorded. Samples were homogenized with Precellys[®] 24 Homogenizer (Bertin Technologies; Paris, France) using 500 μ L of cell lysis buffer (0.05M HEPES, 0.15M NaCl, 0.1M NaF, 0.01M Na₄O₇P₂, 0.0005M EDTA, 0.25M Sucrose, 1mM DTT, 1% Triton-X, 1mM NaVO₄, 1% Protease Inhibitor Cocktail) for gastrocnemius and liver or 300 μ L of cell lysis buffer for adipose tissue. Samples were centrifuged at 13000rpm for 20min at 4 C, and the lysate was collected. The amount of 2-[¹⁴C]-DG was measured as the difference between the specific activity of [¹⁴C] in the total lysate fraction and 2-[¹⁴C]-DG-6-P following lysis and deproteinization.

3.11 Adipose Tissue Macrophage Collection

Adipose tissue macrophages were collected using the EasySep[®] kit for the macrophage marker CD11b (StemCell Technologies; Vancouver, BC). Briefly, adipose tissue was minced and underwent collagenase digestion using Liberase TM Research Grade (Roche Diagnostics; Mannheim, Germany). The cell suspension was filtered using a 100 μ m cell strainer and centrifuged at 500g for 5 minutes. The pellet was re-suspended in recommended medium (2% FBS, 1mM EDTA), and macrophages were sequentially labeled with CD11b+ PE Labeling Reagent, PE Selection Cocktail, and Magnetic Nanoparticles and separated using the EasySep[®] magnet. The final cell suspension was resuspended in 1mL TRIzol[®] Reagent (Invitrogen; Carlsbad, CA) and stored at -80°C until further analysis.

3.12 RNA Isolation and Expression Analyses

RNA analysis was conducted using quantitative PCR to assess the inflammatory profile of macrophages and adipose tissue. Samples of adipose tissue were homogenized in 1mL TRIzol® Reagent (Invitrogen) and previously collected macrophage samples were thawed on ice, and RNA was collected according to the manufacturer's instructions. RNA concentration and quality were assessed using a nanophotometer (Implen Inc.; Westlake Village, CA). Reverse transcription of 1µg of RNA was performed using SuperScript® III (Invitrogen) as per manufacturer's instructions. TaqMan® Gene Expression Assays (Applied Biosystems; Foster City, CA) were used to measure the expression of arginase, CD68, chemokine ligand 1, F4/80, interleukin 1 beta, interleukin 6, inducible nitric oxide synthase 2, ribosomal protein P0, TATA box binding protein, and tumor necrosis factor (Appendix II) using the Rotor-Gene 6000 (Corbett Research; Mortlake, Australia). Gene expression was calculated using the ddCT method (94), in which the expression of a gene of interest is compared to that of the housekeeping gene (ribosomal protein P0 or TATA box binding protein).

3.13 Histology and Immunohistochemistry

Formalin-preserved samples of adipose tissue were paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E) by the Department of Pathology Laboratory Services at McMaster University. Sections of H&E stained adipose tissue were analyzed to determine adipocyte size using ImageJ (NIH; Bethesda, MD) (Appendix III).

In addition, sections of adipose tissue were prepared for immunohistochemistry (IHC) in order to assess macrophage infiltration in the adipose tissue. After dewaxing and rehydrating paraffin-embedded adipose tissue sections, antigen retrieval was performed by boiling the slides in 10mM sodium citrate (pH 6.5) for 15 minutes. Endogenous peroxidase was quenched using a solution of 1% fetal calf serum and 3% hydrogen peroxide in PBS. Subsequently, tissue samples were blocked with 5% normal rabbit serum for 40 minutes, Avidin D (Biotin/Avidin Blocking Kit, Vector Laboratories; Burlingame, CA) for 15 minutes, and Biotin (Biotin/Avidin Blocking Kit, Vector Laboratories) for 15 minutes. Sections were incubated with rat anti-mouse F4/80 (1:100) (AbD Serotec, Oxford, UK) for 2 hours, a biotin-conjugated secondary antibody (1:50) (Vector Laboratories) for 1 hour, and Vectastain ABC solution (Vector Laboratories) for 30 minutes. The DAB Substrate Kit (Vector Laboratories) was used to develop the sections, and slides were counterstained with hematoxylin.

3.14 Statistical Analyses

Analyses between three groups (Chow, HFD Sed, HFD Ex) were analyzed with GraphPad Prism 5 (GraphPad Software Inc.; La Jolla, CA) using 1-way ANOVA with Tukey's multiple comparison post-hoc test. Analyses between two groups (HFD Sed, HFD Ex) were analyzed using a Student's t-test. Glucose and insulin tolerance tests were analyzed using 2-way Repeated Measures ANOVA with Bonferroni multiple comparisons post-hoc test. A p-value less than 0.05 was considered statistically significant. Data are presented as mean \pm SEM.

4. RESULTS

4.1 Pilot Studies

In the first pilot study, the capacity of an acute bout of HIT to activate AMPK was compared to that of endurance exercise in chow-fed mice. HIT phosphorylated both AMPK and the downstream substrate acetyl-CoA carboxylase in the liver to a greater degree than endurance exercise (Figure 4.1A).

The purpose of the second pilot study was to assess the ability of HIT to activate AMPK in the adipose tissue of diet-induced obese mice and to improve insulin sensitivity. A comparison of the pre-exercise glucose tolerance test (GTT) to the post-exercise GTT revealed a significant improvement in glucose tolerance (Figure 4.1B). Similarly, fasting blood glucose dropped following 5 weeks of high-intensity interval training (Figure 4.1C). In addition, Western blotting for AMPK T172 phosphorylation revealed increased activation in the adipose tissue of exercise-trained mice compared to sedentary mice (Figure 4.1D). The results of the pilot study confirmed that high-intensity interval training effectively improves insulin sensitivity and activates AMPK in the adipose tissue of diet-induced obese mice.

4.2 Exercise Capacity

Mice assigned to the HFD Ex group performed baseline and post-exercise exercise capacity tests. Following 6 weeks of high-intensity interval training, time to

exhaustion significantly improved by 50% and maximum speed increased 27% (Figure 4.2).

4.3 Body Mass and Adiposity

High-fat feeding increased body mass by 49%, epididymal fat mass more than 7 fold, adipocyte size more than 4 fold, and percent body fat more than 4 fold in HFD Sed compared to Chow mice. But, importantly, HIT did not alter any of these parameters (Figure 4.3). (Representative images of CT scans and adipocytes can be found in Appendix IV and V). The absence of a change in any measure of body composition or adiposity as a result of the exercise training allows the results to be interpreted independent of the confounding effect of weight loss.

4.4 Food Intake and Substrate Oxidation

As a result of high-fat feeding, food intake decreased by 29% from 9.90 ± 0.37 kcal/day in Chow mice to 7.01 ± 0.79 kcal/day in HFD Sed mice. Despite similar body mass and adiposity to the sedentary group, HFD Ex mice consumed nearly 1.8 times more food per day than HFD Sed mice (Figure 4.4A).

Ambient activity levels are measured as the cumulative number of laser beam breaks in the X, Y, and Z axes over a 24 hour period. The high-fat diet dramatically reduced ambient activity levels, suggesting that, given the reduced food intake in HFD Sed mice, a reduction in ambient activity is the primary mechanism contributing to weight gain. Importantly, exercise training (HFD Ex) increased activity levels by 124%

compared to HFD Sed mice (Figure 4.4B). Therefore, although high-fat feeding reduces ambient activity, exercised animals, even diet-induced obese animals, are significantly more active than sedentary animals.

Consistent with changes in ambient activity levels, VO_2 differed between groups, with Chow and HFD Ex groups having significantly higher VO_2 compared to HFD Sed mice (Figure 4.4C). RER (the ratio of VCO_2/VO_2) is used to calculate non-protein substrate oxidation, and a value of 0.7 indicates 100% fatty acid oxidation while a value of 1.0 indicates 100% carbohydrate oxidation. As expected, the HFD caused a marked drop in RER compared to Chow mice (Figure 4.4D), indicating a much higher utilization of fatty acids as substrate (Figure 4.4E). Importantly, the effect was partially reversed in the HFD Ex group, indicating that they were oxidizing more glucose following exercise training compared to HFD Sed (Figure 4.4 D, E).

4.5 Serum Adipokines

Consistent with increases in adiposity, HFD Sed mice had 30 fold increase in serum leptin levels. Surprisingly, despite similar adiposity as HFD Sed mice, after 6 weeks of HIT, leptin was reduced by 31% in the HFD Ex compared to HFD Sed group (Figure 4.5A).

No differences were observed in the concentrations of the adipokines plasminogen activator inhibitor 1 (PAI1) or IL6 in response to diet or exercise (Figure 4.5B, C). The concentration of serum resistin changed as a result of diet, increasing more than 2 fold with high-fat feeding; however, there was no effect of exercise training on resistin (Figure

4.5D). We also measured TNF α and MCP1, but the concentrations of these cytokines were below the detection limits of our assay.

4.6 Glucose Tolerance and Insulin Sensitivity

4.6.1 Serum Glucose, Insulin, and HOMA-IR

Although fed blood glucose was not significantly different between Chow and HFD Sed groups, fasting blood glucose increased by 66% upon high-fat feeding (Figure 4.6A, B), indicating the development of hyperglycemia with high-fat feeding. Similarly, fasting insulin was also increased more than 5 fold in HFD Sed compared to Chow mice, and HOMA-IR increased almost 8 fold (Figure 4.6C).

Following 6 weeks of high-intensity interval training, fed and fasting blood glucose levels dropped by 15% and 19% in HFD Ex, respectively. Complementary to lower glucose levels, serum insulin and HOMA-IR tended to be reduced in HFD Ex mice but were not statistically significant ($p=0.28$, $p=0.19$, respectively) (Figure 4.6).

4.6.2 Glucose Tolerance Test

Following a 6 hour fast, mice received 1g/kg glucose, and the blood glucose response was evaluated over 120 minutes. The blood glucose response markedly increased with high-fat feeding; but, with exercise, the blood glucose response was lowered, and the response was statistically significant at 120 minutes (Figure 4.7A). This effect is reflected in the area under the curve (AUC), which increased 93% in HFD Sed

mice over Chow mice and dropped by 16% in HFD Ex compared to HFD Sed mice (Figure 4.7B).

4.6.3 Insulin Tolerance Test

To assess the effect of insulin on whole-body glucose clearance, mice received 1U/kg of insulin, and blood glucose response was monitored over the following 120 minutes. Blood glucose was significantly lower in HFD Ex mice compared to HFD Sed mice at 20, 40, 60, 90, and 120 minutes (Figure 4.8A). In addition, the area under the curve (AUC) was reduced by 22% in the HFD Ex compared to HFD Sed group (Figure 4.8B), indicating that the HFD Ex mice had improved insulin sensitivity as a result of high-intensity interval training. Significant differences between groups were observed only when blood glucose values were expressed as a percent of baseline blood glucose, and the difference was not evident when analyzed as absolute blood glucose values.

4.6.4 Hyperinsulinemic-Euglycemic Clamp

The hyperinsulinemic-euglycemic clamp provides the most sensitive measure of insulin action on glucose utilization. The glucose infusion rate (GINF) refers to the rate of glucose required to maintain euglycemia and directly correlates with insulin sensitivity (95). Supporting the results above, GINF dropped by 47% from Chow to HFD Sed mice as a result of high-fat feeding and tended to be rescued by high-intensity interval training ($p=0.09$) (Figure 4.9A). As a result of insulin action, glucose is taken up into target tissues, which is reflected in the glucose disposal rate (GDR). GDR was significantly

lower in the HFD Sed mice compared to Chow mice, dropping by 38%. However, following 6 weeks of high-intensity interval training, GDR increased 19% in HFD Ex mice ($p=0.09$), indicating a tendency for improved response to insulin in muscle and adipose tissue compared to sedentary mice (Figure 4.9B). The hepatic glucose output (HGO) is a measure of glucose production from the liver and is calculated based on the difference between the GDR and GINF. Importantly, HGO is suppressed by insulin, meaning that more insulin sensitive animals will have a lower HGO in response to insulin. Comparing Chow to HFD Sed groups, the HGO was 5 fold higher in HFD Sed mice. In addition, the HFD Ex group showed a very strong trend towards improved insulin sensitivity, as HGO was lowered by 23% compared to HFD Sed mice ($p=0.06$) (Figure 4.9C). Finally, percent suppression reflects hepatic insulin action and the capacity for insulin to suppress basal hepatic glucose production. In support of other parameters measured by the clamp, percent suppression was the greatest in Chow mice, dropped by 37% as a result of the high-fat diet, and increased by 35% with 6 weeks of high-intensity interval training (Figure 4.9D).

4.6.5 Tissue Specific Glucose Uptake

Utilizing 2- ^{14}C -DG, a non-metabolizable glucose analogue, enabled the determination of tissue-specific glucose uptake to the gastrocnemius and adipose tissue. As was reflected in the glucose disposal rate, glucose uptake by the gastrocnemius and adipose tissue was reduced as a result of the high-fat diet but improved following 6 weeks of high-intensity interval training. More specifically, glucose uptake to the gastrocnemius

was reduced by 50% in the HFD Sed mice. With exercise training, glucose uptake to the muscle showed a tendency to improve, increasing 1.5 fold in the HFD Ex group ($p=0.10$) (Figure 4.10A). Similarly, glucose uptake into white adipose tissue was lowered by 59% in HFD Sed compared to Chow mice and increased 2.5 fold in HFD Ex mice (Figure 4.10B). These data indicate that exercise training drastically improved adipose tissue insulin sensitivity but this improvement did not have a dramatic effect on GDR, most likely because the adipose tissue only contributes approximately 20% to insulin-stimulated glucose disposal.

4.7 Adipose Tissue Inflammation and Macrophage Infiltration

4.7.1 Adipose Tissue Macrophage Infiltration

Macrophage infiltration is known to increase as a result of obesity (26, 46, 96). In accordance, macrophage infiltration increased with diet-induced obesity in this study. As measured by quantitative PCR, the expression of CD68 increased more than 10 fold and expression of F4/80 increased more than 9 fold in HFD Sed mice compared to chow-fed controls (Figure 4.11A). Similarly, using immunohistochemistry to assess macrophage infiltration by identifying F4/80+ cells revealed that the percentage of F4/80+ cells increased almost 4 fold from Chow to HFD Sed mice (Figure 4.11B). (Representative images can be found in Appendix VI.)

Many studies suggest that exercise reduces macrophage infiltration into the adipose tissue (76, 97). However, after 6 weeks of high-intensity interval training, neither

the expression of CD68 and F4/80 nor the proportion of F4/80+ cells in the adipose tissue was different when comparing HFD Sed to HFD Ex groups (Figure 4.11A, B).

4.7.2 Adipose Tissue Inflammation

Adipose tissue inflammation was assessed by measuring the expression of inflammatory markers in whole adipose tissue using quantitative PCR. The expression of the genes of interest was compared to that of the housekeeping gene, TATA box binding protein (TBP). High-intensity interval training did not reduce adipose tissue inflammation compared to sedentary controls. The expression of KC, a functional IL8 homolog, and IL6 were not different between HFD Sed and HFD Ex groups. Surprisingly, the expression of TNF α increased 2.3 fold in the adipose tissue of HFD Ex compared to HFD Sed (Figure 4.11C).

4.8 Macrophage Polarization and Inflammatory Markers

4.8.1 Macrophage Polarization

To specifically examine inflammation in adipose tissue macrophages, we isolated macrophages from adipose tissue using a CD11b positive selection kit. Unfortunately, we were not able to examine adipose tissue macrophage inflammation in Chow mice due to insufficient adipose tissue mass required to obtain an adequate number of adipose tissue macrophages. Adipose tissue macrophages tend to shift to a classically activated or M1 polarization in obesity, while exercise causes a shift to an anti-inflammatory state, characterized by alternatively activated or M2 macrophages (26, 46). To assess

macrophage polarization, the expression of arginase and iNOS were investigated. Arginase is an anti-inflammatory marker, characteristic of an alternatively activated or M2 macrophage, while iNOS is a pro-inflammatory marker, characteristic of a classically activated or M1 macrophage. However, mRNA expression of arginase and iNOS in adipose tissue macrophages was not different between HFD Sed and HFD Ex groups (Figure 4.12A).

4.8.2 Macrophage Expression of Inflammatory Markers

In addition, the inflammatory status of adipose tissue macrophages was assessed by measuring the relative expression of TNF α , Il1 β , and KC. However, contrary to the literature that supports an improvement in adipose tissue macrophage inflammation with exercise, but consistent with our findings in whole adipose tissue, there was no difference between HFD Sed and HFD Ex groups in the expression of any of the inflammatory markers (Figure 4.12B).

5. DISCUSSION

Adipose tissue inflammation has been shown to contribute to the development of insulin resistance in obesity (24, 26, 50, 55, 65). Additionally, low-intensity, endurance-type exercise has been associated with ameliorating adipose tissue inflammation in obese mice (75, 76, 97). However, all studies relating exercise-induced improvements in metabolic outcomes to a reduction in adipose tissue inflammation have not ruled out the confounding effect of weight loss (72-76). By utilizing high-intensity interval training, the effect of weight loss was eliminated and allowed for a more complete investigation into the relationship between exercise, adipose tissue inflammation, and insulin sensitivity. Consequently, high-intensity interval training was shown to improve insulin sensitivity independent of changes in both body mass and adipose tissue inflammation.

5.1 Exercise and Insulin Sensitivity

Similar to adaptations observed following low-intensity, endurance exercise (75, 76), high-intensity interval training improved insulin sensitivity in obese mice. In fact, exercise reduced fed and fasting blood glucose by 15% and 19%, respectively. Exercise training also reduced fasting insulin and improved insulin sensitivity as measured by an insulin tolerance test compared to sedentary animals.

Exercise particularly improved hepatic insulin sensitivity, reducing hepatic glucose output and increasing percent suppression of basal hepatic glucose output in exercised mice as compared to sedentary animals. In support of the exercise-induced

improvements in hepatic insulin sensitivity found in this study, Vieira *et al.* (2009) investigated the effect of both a low-fat diet and low-intensity exercise intervention on metabolic complications in obese mice and observed an insulin sensitizing effect as a result of both treatments, but only exercise improved hepatic steatosis.

HIT had only a modest effect on the glucose disposal rate, which is primarily driven (~80%) by glucose uptake into skeletal muscle. In support of the modest effect on GDR, there was a non-significant trend for increased glucose uptake into gastrocnemius in exercise-trained mice. Although glucose uptake is known to increase with exercise (98-100), this study did not observe a significant difference with HIT. The primary fuel source available to the high-fat diet-fed mice is lipid, which is reflected by increased rates of lipid oxidation compared to chow-fed mice. Since lipids are more readily available than glucose, the modest effect of HIT in high-fat diet-fed mice may be reflective of the theory that glucose disposal rate may be based on glucose availability as opposed to glucose metabolism (101). In addition, the effect of exercise on glucose uptake, in particular, may be a more transient effect than its effect on whole-body insulin sensitivity. Changes in glucose transport into skeletal muscle are enhanced during and immediately after exercise (98-100), but the immediate effects of exercise on glucose uptake may differ from the systemic, insulin-sensitizing effects of exercise. Since the hyperinsulinemic-euglycemic clamp was conducted 72 hours after the last bout of exercise, the acute effects of exercise did not confound the results. But, perhaps, a more distinct effect of exercise training on glucose disposal rate would be observed if the clamp were conducted in closer proximity to the last bout of exercise.

5.2 Exercise and Metabolic Flexibility

Metabolic flexibility refers to the capacity to switch between lipid and carbohydrate fuel sources (102). Insulin resistant individuals have impaired metabolic flexibility; and, the diet-induced obese, insulin resistant mice in this study had a low RER, likely due to the consumption of a high-fat diet and suggestive of an impaired capacity to oxidize carbohydrates. But, metabolic inflexibility appears to be rescued with high-intensity interval training by enhancing the ability to oxidize carbohydrate fuel sources in high-fat diet-fed mice. As reflected in a RER value of 0.86 for exercised mice compared with 0.79 for sedentary animals, exercised mice oxidized more carbohydrates and fewer lipids than sedentary animals. Due to the consumption of a diet comprised of 45% fat, the primary substrate available to high-fat diet-fed mice is lipid. Consequently, the increase in carbohydrate oxidation and shift away from primarily lipid oxidation suggests that exercised mice are regaining the capacity to oxidize glucose and demonstrating improved metabolic flexibility. These findings are consistent with a number of human studies that have found that high-intensity interval training elicits adaptations in carbohydrate and lipid metabolism in skeletal muscle that are similar to those observed following endurance training and favour improvements in insulin sensitivity (90, 91, 93).

5.3 The Effect of Weight Loss

Six weeks of high-intensity interval training did not induce weight loss in diet-induced obese mice, as measured by total body mass, epididymal fat mass, adipocyte size, or adiposity. All measures of body mass and adiposity were significantly increased upon high-fat feeding compared with chow-fed mice; however, no difference between sedentary or exercised mice was observed following 6 weeks of exercise training in obese mice.

Interestingly, exercised mice had increased food intake compared to sedentary mice, yet there was no difference in weight. The increase in food intake appears to be compensated by an increase in ambient activity levels and is reflected in an increase in VO_2 compared to sedentary mice.

To date, research has been unable to clearly establish the capacity of exercise to improve metabolic outcomes, including insulin sensitivity and inflammation, independent of weight loss (103-107). Prior to this study, few long-term exercise-intervention studies conducted in rodents had successfully eliminated the confounding effect of weight loss, particularly in studies utilizing models of obesity (72-76). Voluntary and low-intensity exercise in diet-induced obese mice resulted in significant reductions in body weight relative to sedentary controls in studies conducted by Bradley *et al.* and Vieira *et al.* (75, 76). A single study conducted by Kawanishi *et al.* did not elicit weight loss as a result of moderate exercise in diet-induced obese mice; however, while the study showed a reduction in adipose tissue inflammation, no investigation was made into the effects of exercise on insulin sensitivity (97). Although the exercise protocol utilized by Kawaishi

et al. improved adipose tissue inflammation, whether or not it was sufficient to induce improvements in insulin sensitivity remains unknown. Therefore, this study has expanded on previous exercise studies by establishing the effect of exercise on insulin sensitivity and adipose tissue inflammation independent of weight loss. Particularly, utilizing the novel HIT protocol has enabled this study to make more accurate conclusions about the relationship between exercise, insulin sensitivity, and inflammation.

5.4 Exercise, Insulin Sensitivity, and Adipose Tissue Inflammation

In contrast to recent studies conducted in obese mice, this study found that although exercise improves insulin sensitivity, it has no effect on adipose tissue inflammation. Although macrophage infiltration into adipose tissue increased in obese compared to lean mice, no improvement in macrophage infiltration, adipose tissue inflammation, macrophage polarization, or macrophage expression of inflammatory markers was observed following 6 weeks of high-intensity interval training. In fact, the expression of TNF α in adipose tissue was shown to increase with HIT. In contrast, Bradley *et al.* (2008) found 35-77% reductions in the expression of TNF α , MCP1, and PAI1 in the adipose tissue of mice provided access to voluntary exercise on a running wheel as compared to sedentary mice (75). Similarly, Vieira *et al.* (2009) reported significant reductions in MCP1, F4/80, and TNF α in white adipose tissue following 12 weeks of low-intensity exercise (76). While both studies attributed improvements in exercised-induced insulin sensitivity, in part, to the reduction in inflammation, the results

were confounded by the effect of weight loss in the exercised groups. Our data suggest that exercise does not alter inflammation in the absence of weight loss.

Older studies utilizing voluntary exercise that did not induce weight loss in insulin-resistant rats actually found an increase in TNF α expression in the mesenteric adipose tissue. The increase in TNF α expression along with elevated levels of plasma FFA led the authors to hypothesize that exercise-induced TNF α expression in the adipose tissue may promote adipocytolysis and generate FFA to be used by the exercising muscle (108, 109). Our findings agree with these studies as we also observed a greater than 2 fold increase in TNF α with exercise training. Recent studies have demonstrated that lipolysis is the primary cause of macrophage recruitment to adipose tissue (47); therefore, our findings suggest that exercise independent of weight loss, in contrast to our hypothesis, actually promotes adipose tissue inflammation.

Another possibility for why we may not have observed changes in adipose tissue inflammation may be related to the high-intensity exercise protocol. High-intensity or strenuous exercise has been associated with pro-inflammatory effects, often due to tissue or cellular damage (77, 78, 110, 111). In fact, following exhaustive exercise or over-training, adipose tissue expression of TNF α and IL6 has been shown to increase in rodents (77, 78). In addition, pathways implicated in propagating inflammation, such as TLR4 and NF κ B, are activated following over-training (78).

Interestingly, a study utilizing strenuous exercise in rats found an increase in TNF α expression in adipocytes isolated from exercised animals simultaneous with a decrease in soluble TNF α receptors (sTNFR) in the serum. In the serum, TNF α receptors

increase the bioactivity and half-life of the cytokine. Therefore, the local increase in TNF α within the adipose tissue may be paired with a decrease in the effect of TNF α on remote tissues due to the decrease in sTNFR in the serum (109). As a result, high-intensity interval training may have an insulin-sensitizing effect on remote tissues, despite increasing local inflammation in the adipose tissue.

5.5 Exercise-Induced Insulin Sensitivity

Although significant evidence exists for an important role of inflamed macrophages in mediating insulin resistance in obesity (24, 26, 50, 55, 65), the results of this study imply that exercise-induced improvements in insulin sensitivity are independent of alterations in macrophage inflammation. However, Patsouris *et al.* (2008) found that by depleting the population of CD11c+ cells, a marker characteristic of adipose tissue macrophages that express pro-inflammatory cytokines in obesity, the inflammatory status of obese mice was normalized and animals were significantly more insulin sensitive (66). In addition, the role of AMPK in mediating adipose tissue macrophage inflammation recently delineated in the Steinberg lab suggests a strong link between exercise and inflammation, which makes the findings of this study particularly surprising. Taken together, improved insulin sensitivity as a result of reduced adipose tissue inflammation and improved insulin sensitivity as a result of high-intensity interval training appear to be mediated by distinct adaptations, which is consistent with the idea that the causes of insulin resistance are multi-faceted and diverse.

While exercise-induced improvements in insulin sensitivity were found to be independent of changes in adipose tissue inflammation, a variety of other adaptations may be responsible for the insulin sensitizing effects of exercise. Literature supports the hypothesis that improvements in insulin sensitivity are, in part, a consequence of increased glucose uptake into target tissues, particularly skeletal muscle (98, 99). Following exercise, skeletal muscle is more sensitive to the effects of insulin and insulin signaling pathways are activated, ultimately resulting in increased glucose transport into the muscle (100, 112). However, this study observed only a trend towards increased glucose uptake to gastrocnemius in exercised compared to sedentary animals, suggesting that increased glucose uptake to skeletal muscle is not solely driving the improvements in insulin sensitivity.

In addition, due to consumption of a high-fat diet, mice in this study may have had elevated levels of lipid metabolites, both in the circulation or within skeletal muscle, which have been shown to impair insulin signaling and worsen glucose tolerance (113-115). With exercise, fatty acid oxidation in the muscle increases, reducing the presence of fatty acids that interfere with insulin signaling, thus improving insulin sensitivity (116, 117). In addition, obesity is also associated with incomplete fatty acid oxidation, resulting in lipid intermediates that may interfere with insulin signaling (118). Exercise has been shown to tighten the association between fatty acid beta-oxidation and the citric acid cycle, which may reduce the production of intermediates that have the potential to interfere with insulin signaling (119).

As adipose tissue inflammation was assessed exclusively in the epididymal fat pad, a visceral depot, the potential for exercise to reduce inflammation in the subcutaneous depot and thereby exert insulin-sensitizing effects remains possible. However, visceral adipose tissue is known to be more metabolically active and releases free fatty acids into the portal vein, exerting a significant effect on liver metabolism (120-122). In addition, significant evidence indicates that visceral adiposity is a particular risk factor in the development of obesity-associated complications (123-128).

Knowing that the visceral adipose tissue depot is more metabolically active and particularly linked with impaired insulin sensitivity (120, 121, 127), exercise may improve insulin sensitivity by changes in body composition. Although no difference was observed in total body adiposity, exercise may shift the storage of lipids from visceral depots into subcutaneous depots. As a result, the decrease in visceral adipose tissue mass may improve insulin sensitivity.

The liver is emerging as a central player in mediating the insulin-sensitizing effects of exercise. Similar to skeletal muscle, fatty acid oxidation increases with exercise in the liver, reducing the potential interference of lipid intermediates with insulin signaling (129-131). In support of this hypothesis, the decrease in hepatic glucose output and increase in percent suppression measured during the hyperinsulinemic-euglycemic clamp clearly point towards an improvement in hepatic insulin sensitivity with exercise.

5.6 Limitations

A limitation in the study design that could be improved in future studies is exercise-associated stress. Exercise, particularly high-intensity interval training, can cause stress, to which some animals are more sensitive than others. Future studies might consider screening animals and selecting runners that are more comfortable running on the treadmill or measuring cortisol or IgA levels to assess stress.

C57BL/6 mice fed a high-fat diet are particularly susceptible to developing symptoms of the metabolic syndrome, including weight gain, hyperglycemia, hyperinsulinemia, hypercholesterolemia, impaired insulin response, and leptin resistance (132-137). While the C57BL/6 mouse model is currently believed to be the best mouse model of impaired glucose tolerance and type 2 diabetes, there are also limitations in directly translating research findings to a clinical population.

5.7 Future Directions

High-intensity interval training results in improved insulin sensitivity in diet-induced obese mice. This study concludes that exercise-induced improvements are independent of both reduced body mass and adipose tissue inflammation. Moving forward, establishing how and why exercise prevents the development of insulin resistance in obesity is important. To focus these investigations, a better understanding of the time-course of changes in insulin sensitivity in response to high-intensity interval training should be developed. This study utilized a 6 week exercise intervention and found significant improvements in insulin sensitivity, despite observing no change in

adipose tissue inflammation. However, whether insulin sensitivity improved following only 2 weeks of exercise remains unknown.

In addition, exercise may be more effective as a preventative therapy, not applied after the insult of high-fat feeding and development of diet-induced obesity. Therefore, conducting a study in which high-fat feeding and high-intensity exercise begin simultaneously may reveal interesting insights into the efficacy of high-intensity interval training as a preventative therapy as opposed to a treatment, as it was utilized in this study.

Although high-intensity interval training involves significantly less time commitment and energy expenditure than endurance exercise, translating the intensity of the training protocol utilized in this study to a clinical population may not be practical. However, a recent human study suggests that utilizing a lower volume high-intensity interval training protocol involving only 8-12 cycles of intense exercise at 100% $\text{VO}_{2\text{max}}$ is sufficient to elicit favourable metabolic adaptations (138). Therefore, investigating the insulin-sensitizing effects of a shorter HIT protocol in animal studies may allow for the development of a more reasonable exercise protocol that can be more easily translated into an exercise intervention for obese individuals.

Hepatic insulin sensitivity was particularly improved following high-intensity interval training. Therefore, the effects of exercise on liver inflammation and insulin resistance should be investigated. Interestingly, Vieira *et al.* (2009) found that while both low-fat diet and exercise improved high-fat diet-induced insulin resistance and adipose

tissue inflammation, only exercise improved hepatic steatosis, suggesting that exercise-induced metabolic changes may be coordinated by the liver (76).

Delineating the time-course of inflammation during obesity is also an interesting research topic. Although 6 weeks of HIT did not affect adipose tissue inflammation, whether a longer exercise intervention might improve inflammation remains possible. Vieira *et al.* (2009) did not observe any difference in inflammatory markers following 6 weeks of exercise, but found significant reductions in adipose tissue inflammation after 12 weeks of exercise training.

As well, a recent study has shown that macrophage infiltration naturally decreases as the adipose tissue remodels (139). After macrophages have infiltrated the adipose tissue and phagocytosed dead adipocytes, the adipose tissue begins to be repaired, and the number of macrophages decreases. Strissel *et al.* found that following 16 weeks of high-fat feeding, 80% of adipocytes were dead; however, after 20 weeks of high-fat feeding, only 16% of adipocytes were dead, indicating a natural remodeling of the adipose tissue that involves a decrease in macrophage infiltration and pro-inflammatory cytokine expression (139). Whether the insulin-sensitizing effects of high-intensity interval training are maintained, or perhaps enhanced, during the period of adipose tissue remodeling and the natural decrease in inflammation is an interesting future direction.

6. CONCLUSION

High-intensity interval training improves insulin sensitivity in diet-induced obese mice independent of changes in either body mass or adipose tissue inflammation. While both exercise and reduced inflammation are associated with improved insulin sensitivity, this study establishes that the means by which high-intensity exercise improves insulin sensitivity is not via a reduction in adipose tissue inflammation. Practically, high-intensity interval training has the potential to improve metabolic parameters, such as insulin sensitivity, with only minimal time commitment, which is likely to increase exercise adherence and promote good health.

FIGURES

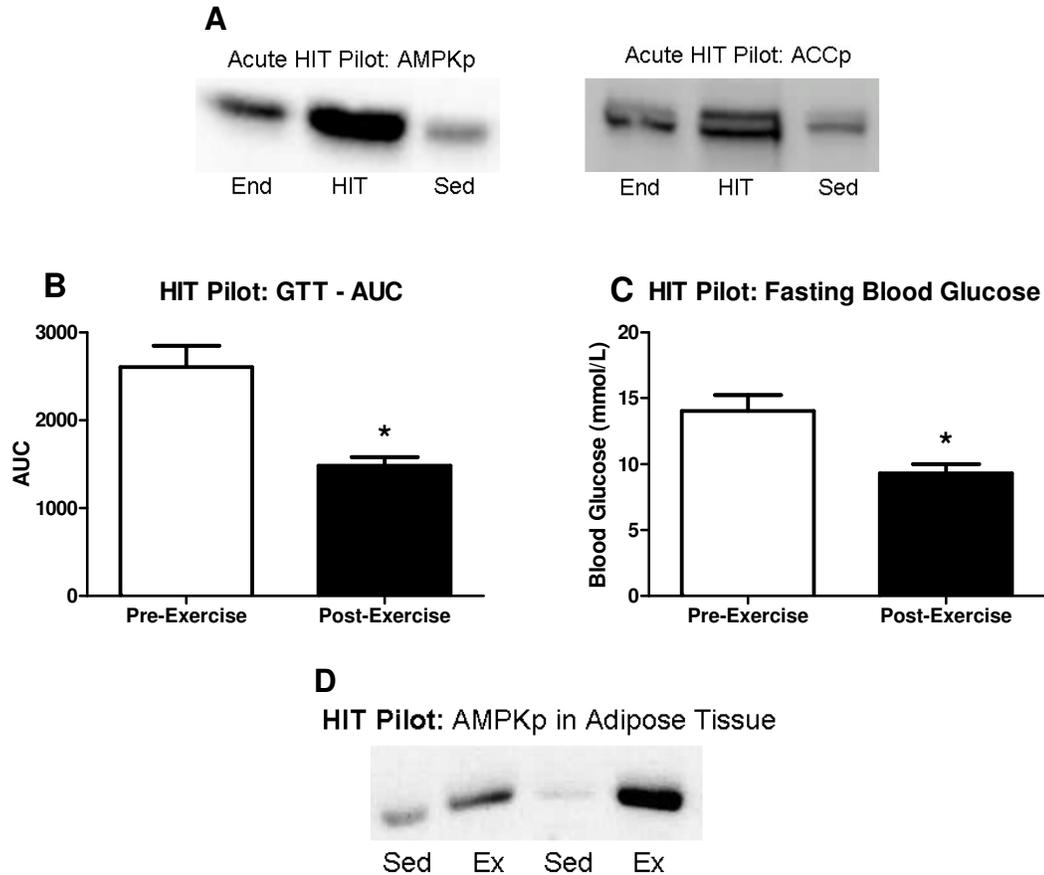


Figure 4.1 High-Intensity Interval Training Pilot Studies (A) A representative immunoblot of phosphorylated AMPK and phosphorylated ACC in liver of endurance exercised (End), HIT exercised (HIT), and sedentary (Sed) chow-fed mice. (B) Glucose tolerance test AUC in pre- and post-exercise ($n=7$) diet-induced obese mice. (C) Blood glucose measured following a 6 hour fast. (D) A representative immunoblot of phosphorylated AMPK in adipose tissue of sedentary (Sed) and exercised (Ex) diet-induced obese mice. Values are mean \pm SEM. AUC: area under the curve. *: $p<0.05$

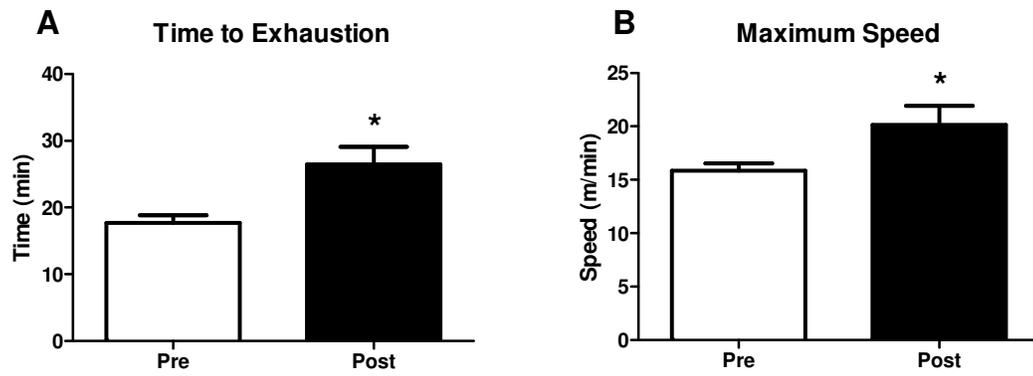


Figure 4.2 Exercise Capacity Exercise capacity, including (A) time to exhaustion and (B) maximum speed, in HFD Ex mice measured pre- and post-exercise ($n=10$). Values are mean \pm SEM. *: $p<0.05$.

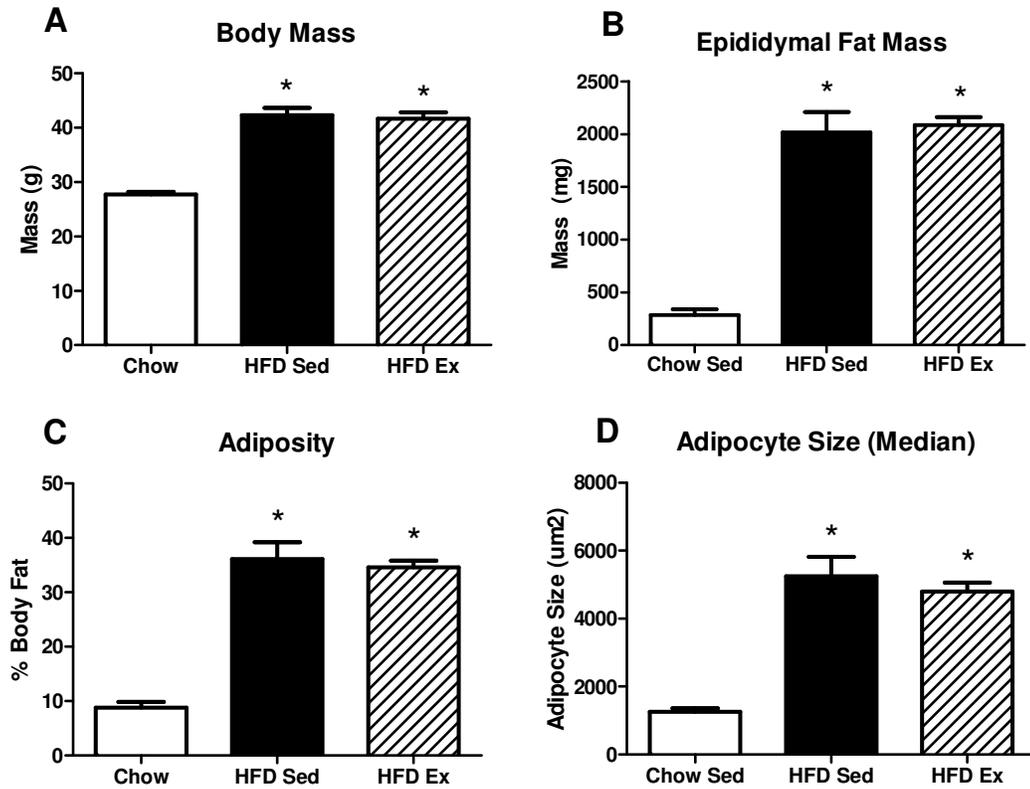


Figure 4.3 Body Mass and Adiposity Measures of body mass and adiposity, including (A) total body mass, (B) epididymal fat mass, (C) adiposity, and (D) median adipocyte size in Chow ($n=14$), HFD Sed ($n=12$), and HFD Ex ($n=8$) mice. Values are mean \pm SEM. *: $p<0.05$ vs Chow.

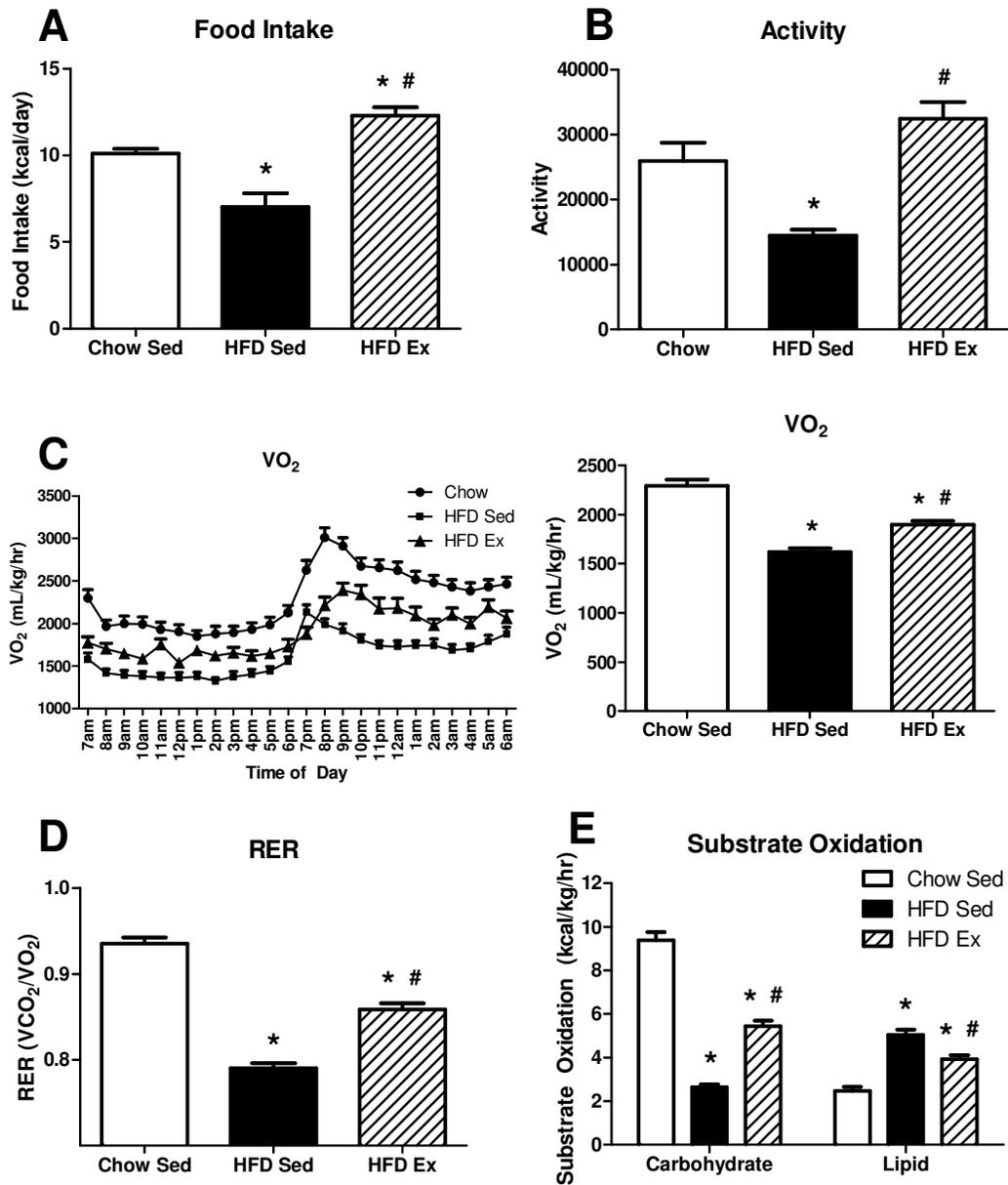


Figure 4.4 Food Intake, Activity, and Substrate Oxidation (A) Daily food intake, (B) ambient activity, (C) VO₂ over 24 hours and daily average, (D) RER, and (E) substrate oxidation, including carbohydrate and lipid oxidation, measured over 72 hours in Chow ($n=16$), HFD Sed ($n=12$), and HFD Ex ($n=8$) mice. Values are mean \pm SEM. *: $p<0.05$ vs Chow; #: $p<0.05$ vs HFD Sed.

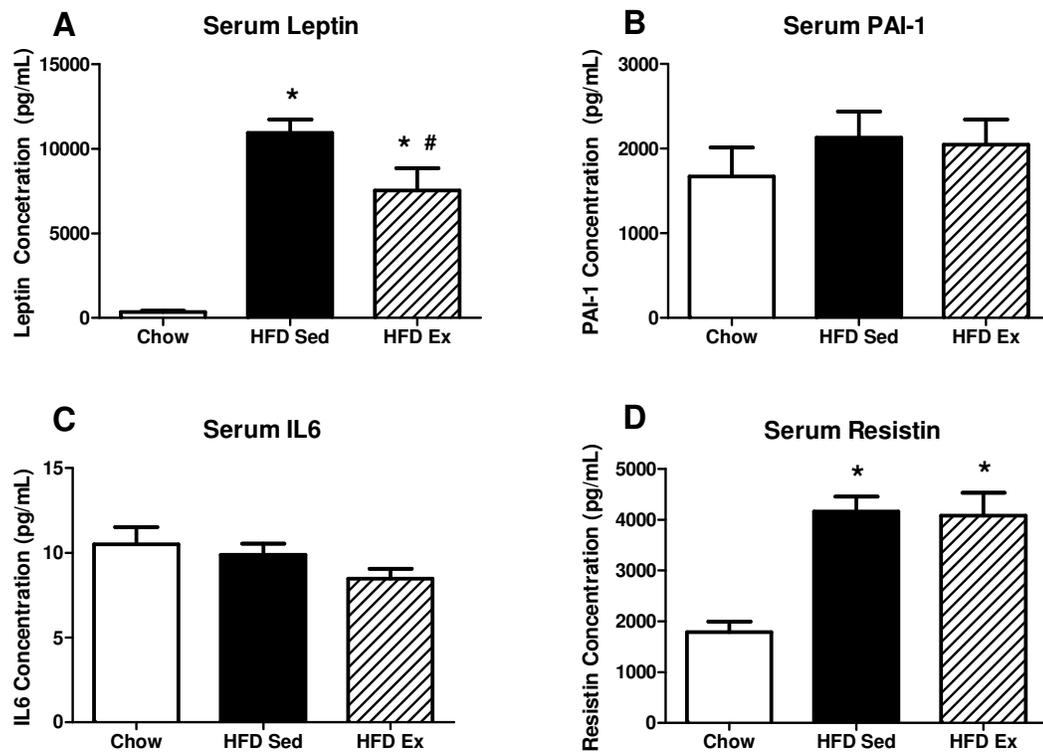


Figure 4.5 Serum Adipokines (A) Leptin, (B) PAI-1, (C) IL6, and (D) resistin in Chow ($n=8$), HFD Sed ($n=10$), and HFD Ex ($n=10$) mice. PAI-1: plasminogen activator inhibitor -1; IL6: interleukin 6. Values are mean \pm SEM. *: $p<0.05$ vs Chow; #: $p<0.05$ vs HFD Sed.

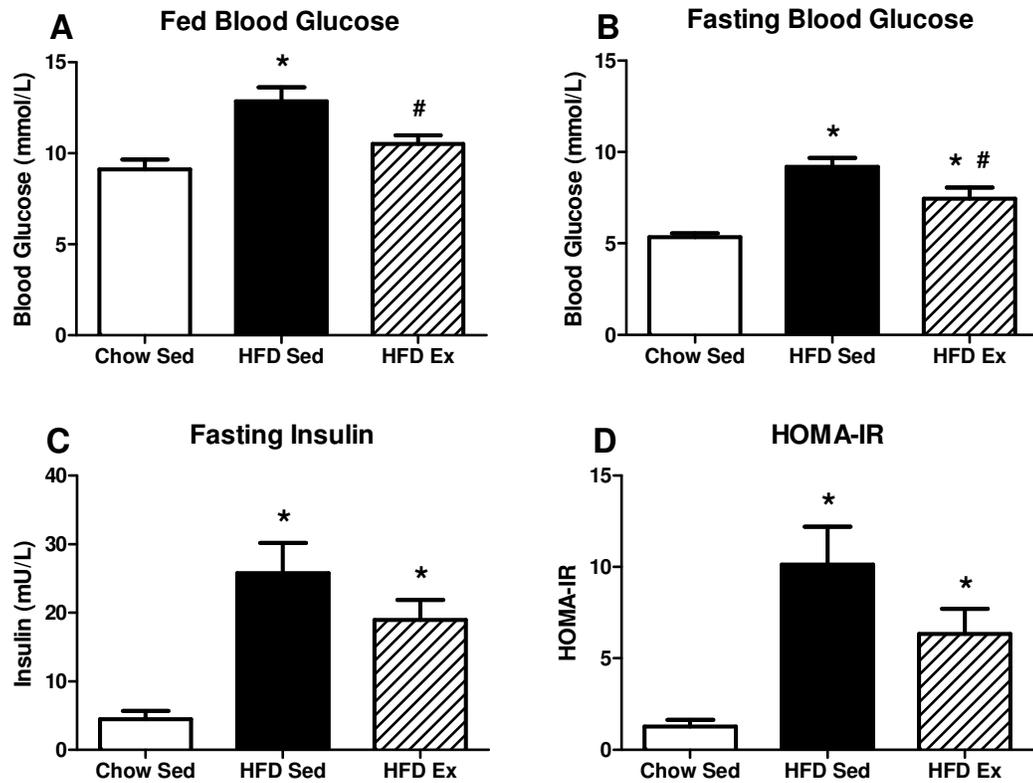


Figure 4.6 Serum Glucose, Insulin, and HOMA-IR (A) Fed blood glucose, (B) fasting blood glucose, (C) fasting insulin, and (D) calculated HOMA-IR in Chow ($n=7$), HFD Sed ($n=9$), and HFD Ex ($n=6$) mice. Values are mean \pm SEM. *: $p<0.05$ vs Chow; #: $p<0.05$ vs HFD Sed.

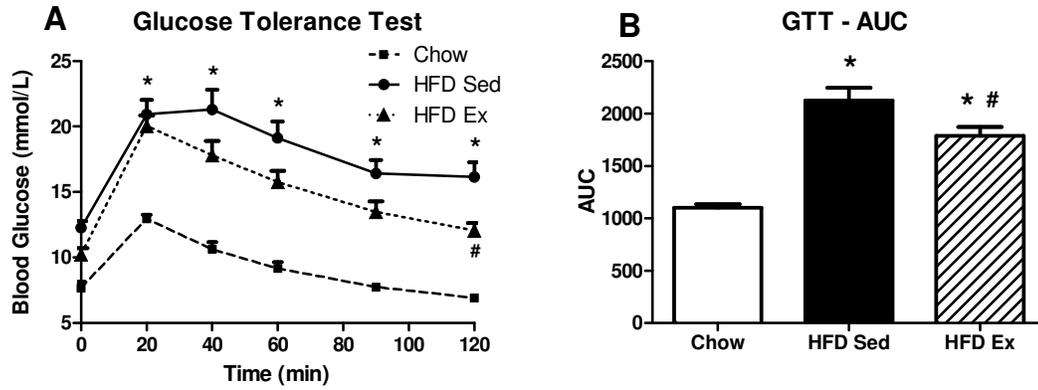


Figure 4.7 Glucose Tolerance Measured by Glucose Tolerance Test (A) Blood glucose response and (B) AUC in Chow ($n=5$), HFD Sed ($n=9$), and HFD Ex ($n=11$) mice in response to 1g/kg glucose delivered by intraperitoneal injection following a 6 hour fast. AUC: area under the curve. Values are mean \pm SEM. *: $p<0.05$ vs Chow; #: $p<0.05$ vs HFD Sed.

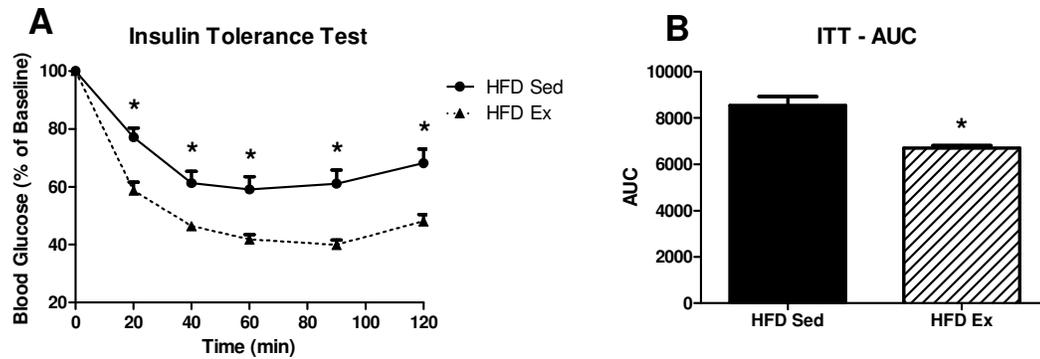


Figure 4.8 Insulin Sensitivity Measured by Insulin Tolerance Test (A) Blood glucose response and **(B)** AUC in HFD Sed ($n=13$) and HFD Ex ($n=11$) mice in response to 1U/kg insulin delivered by intraperitoneal injection following a 6 hour fast. AUC: area under the curve. Values are mean \pm SEM. *: $p<0.05$.

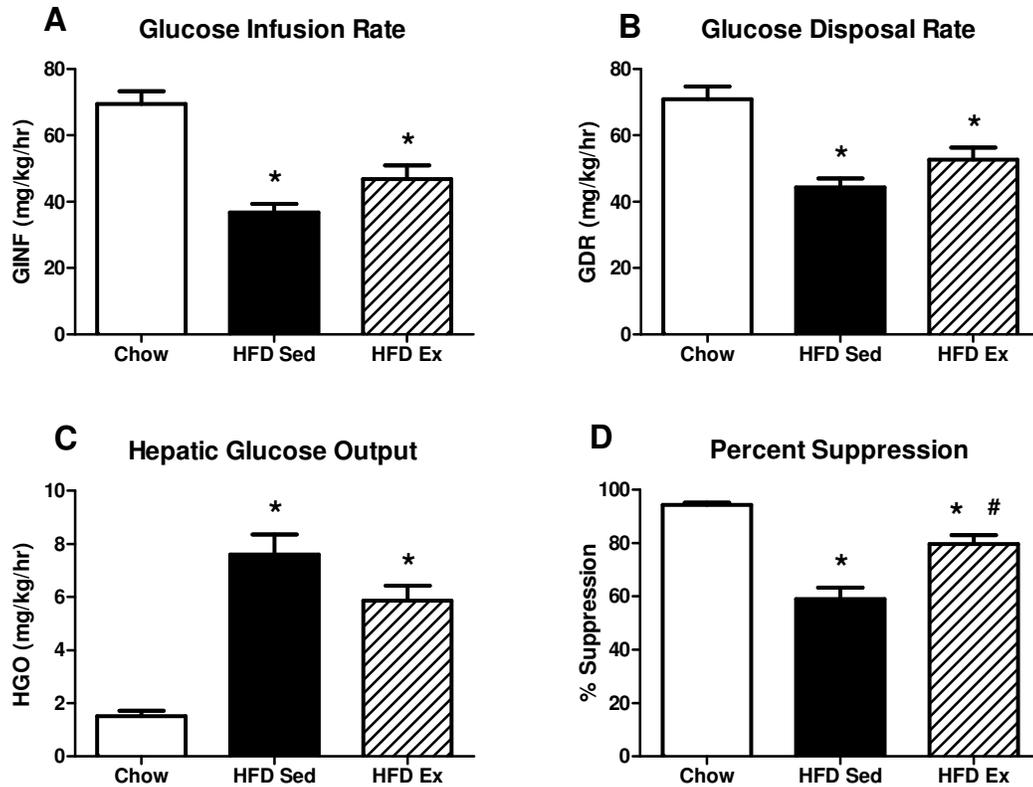


Figure 4.9 Insulin Sensitivity Measured by Hyperinsulinemic-Euglycemic Clamp (A) Glucose infusion rate, (B) glucose disposal rate, (C) hepatic glucose output, and (D) percent suppression measured in Chow ($n=7$), HFD Sed ($n=7$), and HFD Ex ($n=8$) mice. Values are mean \pm SEM. *: $p<0.05$ vs Chow; #: $p<0.05$ vs HFD Sed.

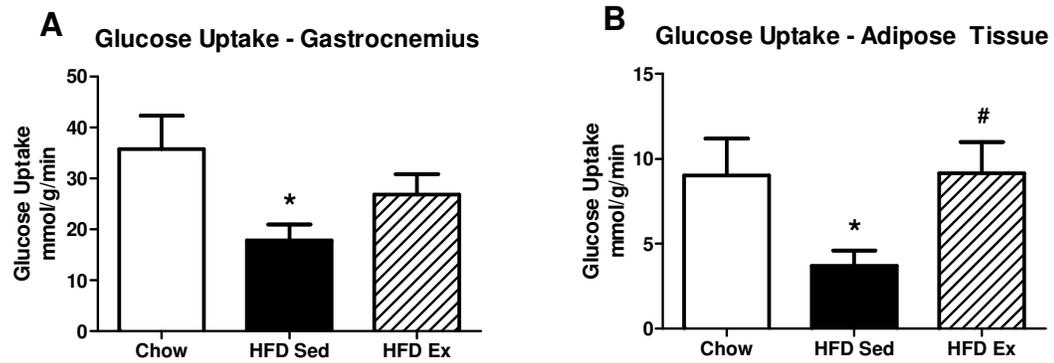


Figure 4.10 Tissue-Specific Glucose Uptake Insulin-stimulated glucose uptake to (A) gastrocnemius and (B) adipose tissue measured following hyperinsulinemic-euglycemic clamp in Chow ($n=7$), HFD Sed ($n=7$), and HFD Ex ($n=7$) mice. Values are mean \pm SEM. *: $p<0.05$ vs Chow; #: $p<0.05$ vs HFD Sed.

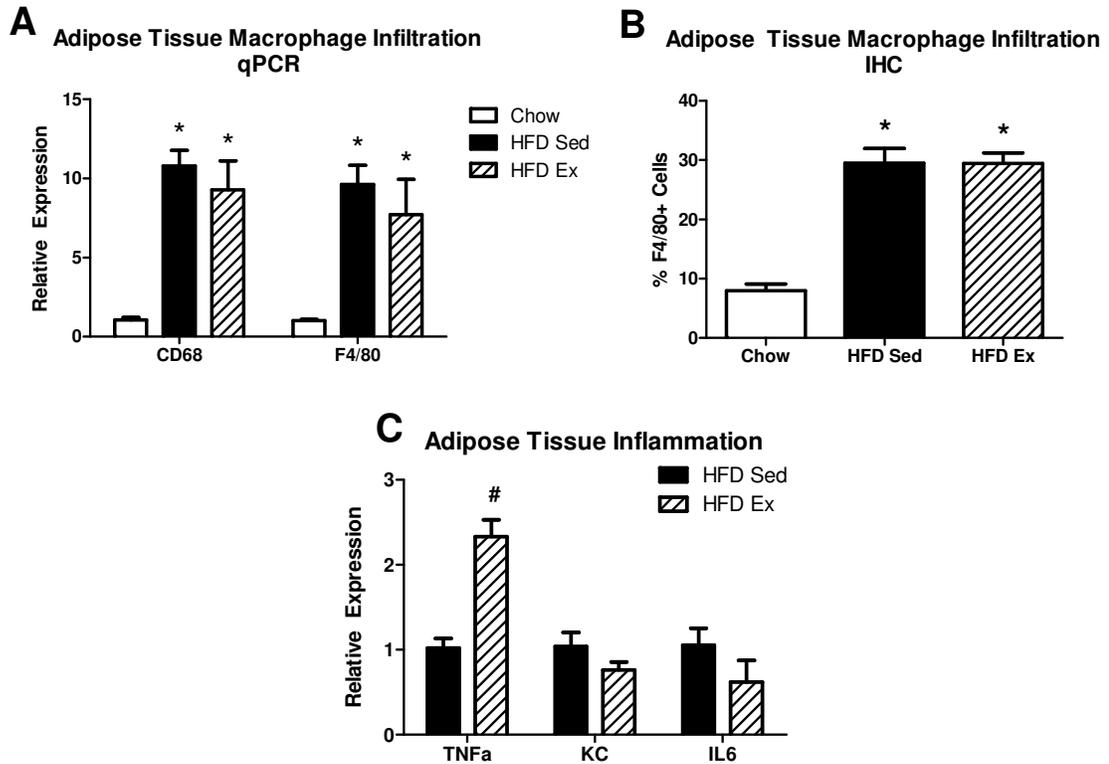


Figure 4.11 Inflammation and Adipose Tissue Macrophage Infiltration Macrophage infiltration into adipose tissue assessed by (A) quantitative PCR and (B) immunohistochemistry in Chow ($n=6$), HFD Sed ($n=5$), and HFD Ex ($n=5$) mice. (C) Relative expression of inflammatory cytokines, TNF α , KC, and IL6, measured in adipose tissue by qPCR in HFD Sed ($n=5$) and HFD Ex ($n=5$) mice. TNF α : tumor necrosis factor α ; KC: chemokine (C-X-C motif) ligand 1; IL6: interleukin 6. Values are mean \pm SEM. *: $p<0.05$.

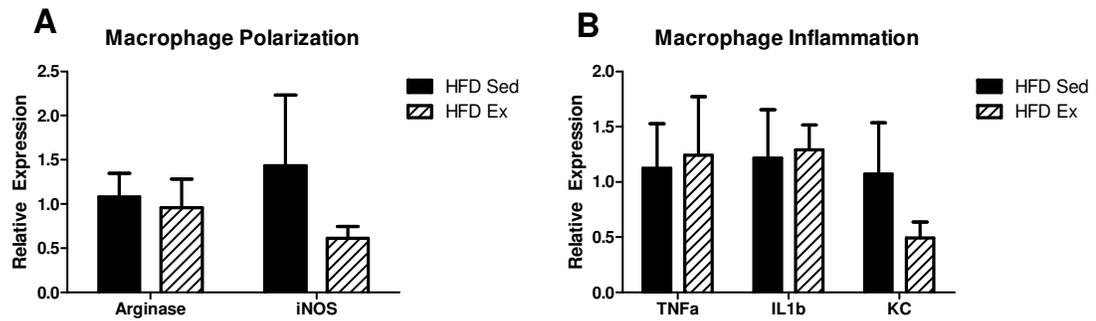


Figure 4.12 Adipose Tissue Macrophage Polarization and Inflammation (A) Adipose tissue macrophage polarization assessed by relative expression of arginase and iNOS in HFD Sed ($n=3$) and HFD Ex ($n=4$) mice. (B) Relative expression of adipose tissue macrophage inflammatory cytokines, TNF α , IL1 β , and KC, in HFD Sed ($n=3$) and HFD Ex ($n=4$) mice. iNOS: inducible nitric oxide synthase; TNF α : tumor necrosis factor α ; IL1 β : interleukin 1 β ; KC: chemokine (C-X-C ligand) motif. Values are mean \pm SEM.

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APPENDIX I: Adipose Segmentation with Amira

1. Open image on Amira.
2. Data should be “Short,” “512x512x512,” and “Little Endian.”
3. Select data object from object pool.
4. Select Properties → Digital Filters and set to “Guassian,” “3D,” “Sigma=0.67x0.67x0.67,” and “Kernel=3x3x3.”
5. Select data object from object pool and select Labelling → Labelfield.
6. Select Zoom and Data Window and set slider values to -1024 to 2048.
7. In toolbox panel, select “Magic Wand” and enable “All Sizes” and “Same Material Only.” Set range from -500 to 1000.
8. In “Selection” area, press “+” to add selection as a new material.
9. Go to Segmentation → Fill Holes → All Slices.
10. Select “Pick&Move” and enable “All Slices.” Go to Selection → Shrink → All Slices. Repeat. Add new selection volume to a new material. Delete the old material by pressing “-.”
11. Locate the lungs/trachea. Select “Magic Wand” and set range from -1000 to -250 and click on a portion of the lungs/trachea. Add new selection volume to a new material.
12. Select “Pick&Move” and enable “All Slices.” Go to Selection → Grow → Volume. Repeat. Add new selection volume to lung material.
13. In toolbox panel, select “Threshold” and enable “All Slices” and “Current Material Only.” Set range from -450 to -165. Add selected volume to a new material (this is body adipose).
14. Make a histogram and import data to Excel.
 - a. Select data object from object pool and select Measure → Histogram.
 - b. Select white box on left of histogram object and select Labels.
 - c. Drag blue connection line to Labelfield and click to attach.
 - d. Click on histogram object.
 - e. In properties box, set range to -500 to 100 and number of bins to 256. Set to “Absolute” and “Logarithmic.”
 - f. Select “Apply.”
 - g. Save histogram as .csv and export to excel.

APPENDIX II: TaqMan® Gene Expression Assays

Gene Name	Assay ID
Arginase	Mm01190441_g1
Carnitine palmitoyltransferase 1a	Mm00550438_m1
CD68 antigen	Mm00839636_g1
Chemokine (C-X-C motif) ligand 1	Mm00433859_m1
Citrate synthase	Mm00466043_m1
EGF-like module containing, mucin-like, hormone receptor-like sequence 1	Mm00802529_m1
Hydroxyacyl-Coenzyme A dehydrogenase	Mm00492535_m1
Interleukin 1 beta	Mm00434228_m1
Interleukin 6	Mm00446190_m1
Nitric oxide synthase 2, inducible	Mm00440502_m1
Ribosomal protein, large, P0	Mm01974474_gH
TATA box binding protein	Mm00446973_m1
Tumor necrosis factor	Mm00443258_m1

APPENDIX III: Adipocyte Size Measurement

Imaging Adipose Tissue:

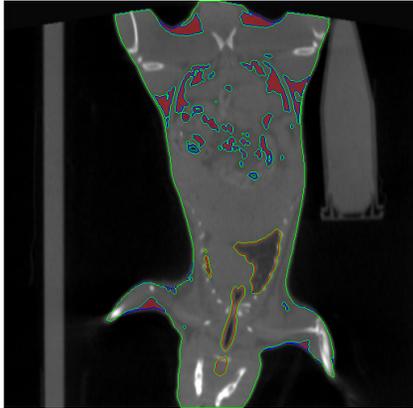
1. Turn on microscope (Nikon Eclipse 90i, Nikon; Tokyo, Japan), both cameras, and computer.
2. Open NIS Elements AR (Nikon) program.
3. Select DS-SMc camera.
4. In 90i panel, ensure light, camera, and filter are set to Bright Field. Adjust image quality to maximum values.
5. Click “AutoWhite” while focused on blank area of slide. (If contrast is not ideal, adjust “Exposure” and “Gain” manually.) Record White Balance settings.
6. Examine adipose tissue using 10X objective.
7. Save images as tif files. Record um/px.

Adipocyte Size Measurement:

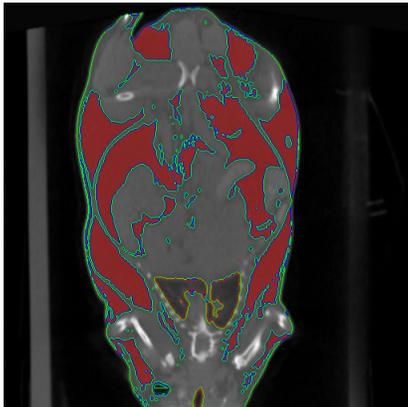
1. Open image with ImageJ.
2. Go to Analyze → Set Scale: enter “Distance in Pixels” and “Known Distance” as measured on microscope, select “Global.”
3. Circle adipocyte using “Freehand Selections” tool.
4. Go to Analyze → Tools → ROI Manager, click “Add” and select “Show All.”
5. Go to Analyze → Measure.
6. Repeat steps 3-5 for each adipocyte.
7. Area measurements are recorded in Results box.

Appendix IV: Representative CT Images

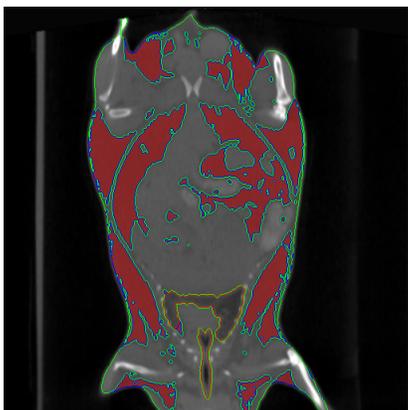
Chow:



HFD Sed:

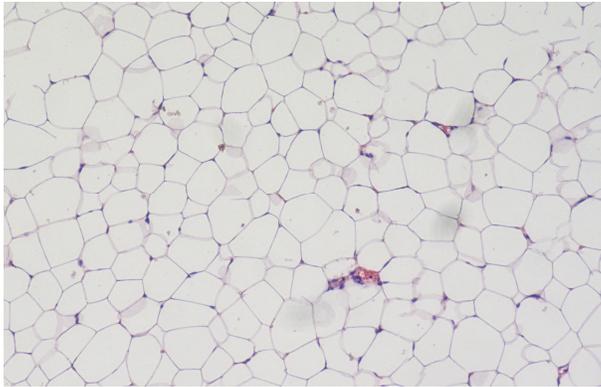


HFD Ex:

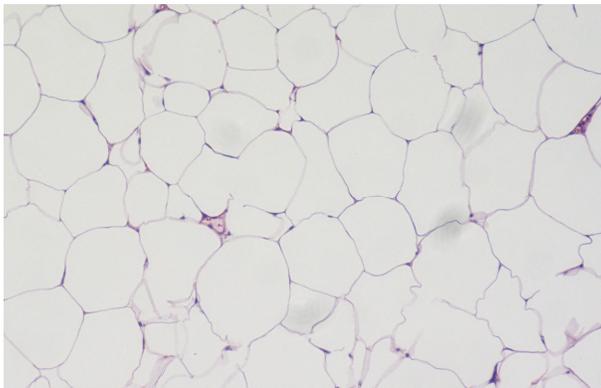


Appendix V: Representative H&E Images of Adipose Tissue

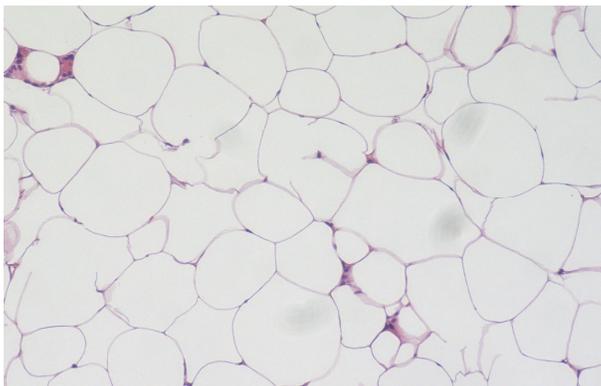
Chow:



HFD Sed:

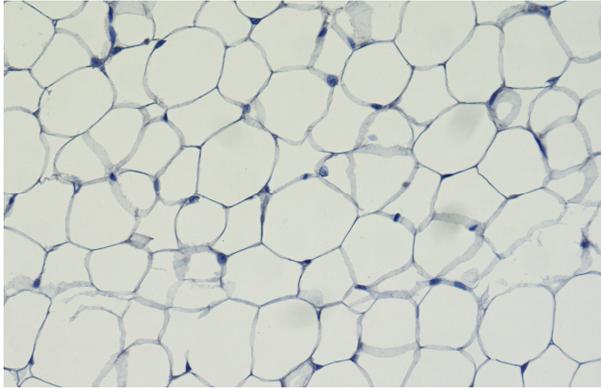


HFD Ex:

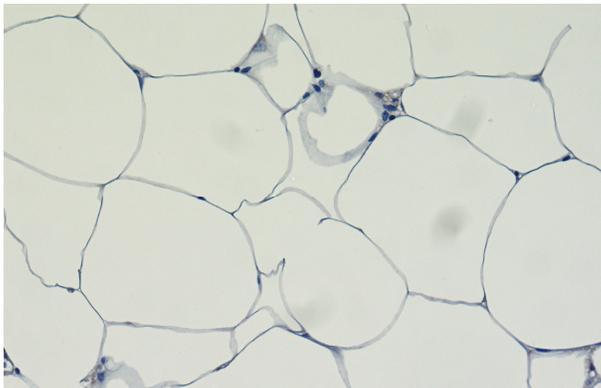


Appendix VI: Representative IHC Images of F4/80 in Adipose Tissue

Chow:



HFD Sed:



HFD Ex:

