HEPATIC TRIGLYCERIDE METABOLISM IN RESPONSE TO ACUTE AND CHRONIC EXERCISE: A TALE OF TWO INTENSITIES

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A Dissertation submitted to the

Graduate School of New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Nutritional Sciences

Written under the direction of

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New Brunswick, New Jersey

May 2015

ABSTRACT OF THE DISSERTATION

Hepatic Triglyceride Metabolism in Response to Acute and Chronic Exercise:

A Tale of Two Intensities

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Impaired hepatic triglyceride (TG) metabolism is associated with dysfunctions such as insulin resistance and elevated VLDL-TG secretion. Chronic exercise lowers plasma TG and hepatic TG but many benefits of chronic exercise are due to repeated effects of single exercise sessions. Little is known, however, about effects of acute exercise on hepatic TG metabolism or the influence of exercise intensity in males vs. females. We examined impacts of single bouts of moderate-intensity continuous exercise (CE) vs. high-intensity interval exercise (HIIE) on hepatic TG metabolism and secretion in mice of both sexes. Hepatic TG was transiently increased on the day of exercise and in females the increase was greater with HIIE. These exercise-related

changes appeared driven by enhanced transcription of the lipid-droplet coating protein Perilipin 2. On the day after exercise, VLDL-TG secretion rate was only reduced by HIIE in females. These findings demonstrated intensity-dependent and sex-specific effects of acute exercise.

Because of our findings that single bouts of HIIE alter hepatic TG metabolism more than CE and the potential for chronic exercise to attenuate severity of complications associated with menopause, we compared 6 weeks of training using these exercise types on hepatic TG metabolism and secretion, glucose tolerance, body composition, and strength in ovariectomized (OVX) and sham-operated (SHAM) mice. Additionally, we measured energy expenditure and substrate oxidation during and immediately after exercise and assessed post-exercise spontaneous physical activity (SPA) to further characterize these exercise modalities. In OVX and SHAM, CE and HIIE elicited similar energy expenditures during exercise and in the post-exercise period lowered absolute carbohydrate oxidation and SPA. OVX vs. SHAM displayed impaired glucose tolerance, elevated blood glucose, and greater body fat despite lower hepatic TG, as well as lower strength, and these outcomes were not affected by training. In contrast to responses to single exercise sessions, chronic HIIE increased hepatic AMPK protein. Further, the reduction in VLDL-TG secretion after a single HIIE session was not maintained after training. These results reveal intensity-dependent effects of habitual exercise on hepatic AMPK expression and with our findings of single bouts reveal distinct responses in hepatic TG metabolism to acute vs. chronic exercise.

Acknowledgements

I would like to first thank Drs. Gregory C. Henderson and Sara C. Campbell for the mentoring they've given me over the past several years. Thank you Greg for all the help and advice you have provided and continue to provide. I accomplished so much and learned many critical skills under your guidance. Thank you Sara for taking on the role of advisor, performing hours and hours of surgeries that were critical for my research, and all the support and advice you've given me.

Thank you to my committee members Drs. Tracy G. Anthony, Dawn Brasaemle, Ken McKeever, and Shawn Arent for suggestions for improving my research. Also, thank you Tracy for allowing me to do work in your lab.

Thank you to Keith Anacker, Dylan Klein, Taylor McConnell, Kristen Rizkalla, Daphne Bienkiewicz, Zunaira Khan, and Jesse Liou for the tremendous amount of help in the lab and animal facility, especially during the many weekends and holidays.

Thank you to the Department of Nutritional Sciences, Department of Exercise Science, Rutgers Center for Lipid Research, and the Institute for Food Nutrition and Health for support, advice, and financial assistance for attending conferences. Thank you to Dr. Sue Shapses for encouragement and advice and Drs. Judith Storch and Dawn Brasaemle for guidance as graduate program directors.

Thank you to Drs. Malcolm Watford, Joseph Dixon, Paul Breslin, Daniel Hoffman, Judith Storch, Sue Shapses, and Ariel Igal for excellent course instruction.

Thank you to graduate students, postdocs, and staff, including Dr. Angela Gajda, Dr. Pat Davitt, Matt Kochem, Anna Dinh, Juliet Gotthardt, Lindsey Phillipson-Weiner, Dr. Claudia Pop, Emily Mirek, Dr. Gabe Wilson, Sara Hassanien, Peggy Policastro and Mayda Hernandez for support. Thank you to Nanci Rossi and staff of the Bartlett Animal Facility.

Thank you to Drs. Adrianne Bendich and Ronit Zilberboim for wonderful mentoring at GlaxoSmithKline and pushing me to pursue a PhD.

Finally, thank you to Drs. Gregg Transue and Monica Torres from the Division of Life Sciences. Through working with you, I developed essential teaching skills and discovered a new joy in teaching science.

Dedications

To my loving parents, Renato and Carmen Tuazon. Thank you for always emphasizing the importance of learning and for doing everything possible to make sure I received a proper education. Pa, thank you for always allowing me to use your tools to take apart things in the house to understand how they operated. Mom, thank you for letting me stick my pet goldfish in the freezer to see if cryogenic preservation might work. You both cultivated a deep curiosity and passion for discovery within me that is still growing today. I hope I can give Maya that same wonderful gift.

To my brother, Dr. JV Tuazon. Thanks for being a role model. Your courage to pursue all your dreams and to be the first in our family to obtain a doctoral degree was a huge inspiration.

To my mother-in-law, Linda La Santa. Thank you for the many long hours you spend taking care of Maya. I don't know how Lauren and I would balance everything without your never ending help.

To my beautiful daughter, Maya Lynn "Myelin Sheath" Tuazon. You were born during the busiest and most stressful possible period of my research. Thank you though for not being born during the middle of any experiments. You provide extra motivation behind everything I do now and I hope to inspire you to achieve your dreams, no matter how difficult, crazy, and scary they may seem.

To my amazing wife, Lauren La Santa. You deserve the biggest thanks. You always gave me courage when things were at its most difficult. From the very

beginning, when it seemed like the end was never in sight, until the very end, you made endless sacrifices to see me through. I certainly couldn't have completed this long journey without you. Thank you and I love you.

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List of Abbreviations

ACC	Acetyl-CoA Carboxylase
AMP	Adenosine Monophosphate
AMPK	AMP-Activated Protein Kinase
ANCOVA	Analysis Of Covariance
ANOVA	Analysis Of Variance
ATGL	Adipose Triglyceride Lipase
ATP	Adenosine Triphosphate
AUC	Area Under The Curve
BW	Body Weight
CE	Continuous Exercise
CON	Sedentary Control
CPT1	Carnitine Palmitoyl Transferase-1
CREB	cAMP Response-Element Binding Protein
CVD	Cardiovascular Disease
FA	Fatty Acid
FFA	Free Fatty Acid
FFM	Fat Free Mass
FGF21	Fibroblast Growth Factor 21
HIIE	High-Intensity Interval Exercise
HSL	Hormone-Sensitive Lipase
IMTG	Intramuscular Triglyceride
LD	Lipid Droplet
LPL	Lipoprotein Lipase
LSD	Least Significant Difference
MCD	Malonyl-CoA Decarboxylase
MTP	Microsomal Triglyceride Transfer Protein
OGTT	Oral Glucose Tolerance Test
OVX	Ovariectomized

PLIN2	Perilipin 2
PPARα	Peroxisome Proliferator-Activated Receptor-α
RER	Respiratory Exchange Ratio
SHAM	Sham-Operated
SPA	Spontaneous Physical Activity
TG	Triglyceride
VCO_2	Carbon Dioxide Production
VLDL	Very-Low Density-Lipoprotein
VLDL-TG	Very-Low Density-Lipoprotein-Triglyceride
VO_2	Oxygen Consumption
VO_{2peak}	Peak Oxygen Consumption

Chapter 1: Introduction and Literature Review

Introduction

Excess triglyceride (TG) accumulation in the liver affects 27-34% of the general population (150) and is associated with insulin resistance (33, 66, 118) and increased hepatic secretion rate of TG into the blood as very-low density-lipoprotein-TG (VLDL-TG) (33). Blood TG when elevated increases coronary heart disease risk (4), and in the fasted state circulates primarily in the form of VLDL-TG. Furthermore, high hepatic TG content can progress to the more pathological forms which could possibly lead to liver failure (150). Strategies are thus needed to improve hepatic TG metabolism in order to prevent excess TG in the liver and to lower VLDL-TG secretion.

Exercise is an attractive intervention strategy as chronic exercise lowers hepatic TG content (128, 149) and VLDL-TG secretion (137). However, the effects of single exercise bouts likely also play a major role in the benefits of chronic exercise training (29, 53, 55). In addition, exercise intensity may influence the magnitude of exercise effects. Therefore, further understanding of the acute effects of single exercise bouts vs. chronic exercise-induced adaptations, as well as of the relative efficacy of exercise modalities of different intensities, could lead to improved exercise prescription for improving hepatic TG metabolism and ultimately overall health.

Overview of hepatic TG metabolism and VLDL-TG secretion

TGs are synthesized by the esterification of three fatty acids (FAs) to a glycerol phosphate backbone. Sources of FAs can be of endogenous (de novo synthesized) or

exogenous (dietary) origin. Endogenously, FAs are synthesized through the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC), which exists as two isoforms (ACC1 and ACC2), to form malonyl-CoA (126). Malonyl-CoA is then used as substrate for synthesis of FAs by fatty acid synthase. Within the intestinal lumen, dietary TG is hydrolyzed, releasing FAs for uptake by enterocytes. These FAs are re-esterified into TG in the enterocyte and enter the circulation on chylomicron particles (140). Chylomicron-TGs are then hydrolyzed by lipoprotein lipase (LPL) localized to the capillary endothelium of peripheral tissues, such as muscle, heart, and adipose, followed by uptake of released FAs by these tissues with some spillover of FAs into the circulation (127). Lipid uptake by the liver can occur by receptor-mediated endocytosis of lipoprotein particles or by uptake of plasma free FAs (FFAs) derived from adipose lipolysis, TG degraded by hepatic lipase, or spillover (13). Within the cytosol, TGs are stored in lipid droplets (LDs) surrounded by LD-associated proteins that regulate lipolysis by modulating access of the lipases hormone-sensitive lipase (HSL) (130) and adipose triglyceride lipase (ATGL) to substrate lipids (77) . In liver, TG can be used as a source of FAs for oxidation or released into the blood as part of very-low density-lipoprotein (VLDL) particles. For the production and secretion of VLDL, cytosolic TG undergoes lipolysis and FAs are transported into the endoplasmic reticulum, re-esterified into TG, and then through microsomal triglyceride transfer protein (MTP) incorporated into VLDL and secreted into the blood (86). In the capillary lumens of peripheral tissue, VLDL-TGs are hydrolyzed by LPL and the released FAs are taken up by surrounding tissues.

Because of the uptake of FAs derived from TG in VLDL particles by multiple extra-hepatic tissues, the liver plays a central role in whole-body lipid trafficking.

Elevated hepatic TG content is associated with insulin resistance (33, 66, 118). Additionally, increased concentration of plasma TG, circulating in the fasted state primarily as VLDL-TG, is associated with increased coronary heart disease risk (4). The concentration of VLDL-TG in the blood is not only determined by rate of clearance in the circulation, but also by rate of secretion by the liver. Thus, targeting hepatic TG metabolism can be a potential strategy for reducing risk of cardiovascular disease (CVD). Further, if accelerated, VLDL-TG secretion can potentially lead to increased adiposity through delivery of lipid to adipose and increase risk of insulin resistance through promoting lipid accumulation in muscle (104). Indeed, mouse models have demonstrated that the majority of FA flux into adipose tissue is derived from plasma TG rather than FFA (144), and alterations in hepatic lipid metabolism (liver specific knockdown of stearoyl-CoA desaturase-1) can protect against adiposity (99) and improve whole-body glucose tolerance (36). Dysregulated hepatic TG metabolism therefore can possibly play a central role in the development of obesity and associated chronic diseases such as CVD and Type 2 Diabetes. Exercise lowers both hepatic TG content (128, 149) and VLDL-TG secretion (137), but the relative effectiveness of exercise protocols of different intensities is largely unknown.

High-intensity interval exercise vs. moderate-intensity continuous exercise

High-intensity interval exercise (HIIE), which involves alternating between relatively easy and challenging intensities within a single exercise bout, may be a more effective means of improving many aspects of health than traditional moderate-intensity continuous exercise (CE) at steady workload (i.e., jogging at constant speed) because of the potential effects of the repeated short periods of high intensity exertion. Chronic HIIE has been shown to promote fat loss to a greater extent than CE despite similar (135) or lower (136) energy expenditures, suggesting an important role of intensity in improving body composition. Mitochondrial enzyme activity (44) and abundance (80) are also increased in skeletal muscle with chronic HIIE (44, 80). As well, single sessions of HIIE are capable of activating signaling pathways involved in mitochondrial biogenesis in skeletal muscle (7, 79). Importantly, HIIE is perceived to be more enjoyable than CE (6) and thus may be relatively more effective at maintaining exercise program adherence in certain populations. Collectively, it therefore appears that HIIE could generally be of higher utility than CE in certain cases for improving whole-body lipid metabolism and body composition. Despite the emerging work on HIIE in muscle however, little is known about the impacts of this exercise modality on hepatic triglyceride metabolism and secretion.

Continuous exercise and hepatic TG metabolism

Both plasma TG (30) and hepatic TG content (128) decrease with chronic endurance exercise. However, much of the effect of chronic exercise on TG metabolism

is likely due to acute impacts of repeated single exercise bouts rather than training-induced adaptations per se. This is demonstrated by large increases in fasting plasma TG concentrations in trained individuals when habitual training ceases (49) and the lack of differences between trained and untrained individuals (56) 60 hours after the most recent exercise bout. Although increases in activity of LPL induced by single sessions of exercise (117) may contribute to reduced TG by enhancing clearance even in the absence of a reduced hepatic VLDL-TG secretion rate post-exercise, studies in rats show chronic running wheel access reduces VLDL-TG secretion (100, 121). Thus, clearance is not the only aspect of VLDL-TG kinetics that is apparently regulated by exercise participation. It is unknown how to relate running wheel access to a well-defined exercise prescription because exercise intensity is uncontrolled in wheel running, so it remains unclear how to design discrete exercise sessions in order to achieve a reduction in VLDL-TG secretion. There was some initial evidence in a human study that single bouts of moderate-intensity CE may reduce the rate of VLDL-TG secretion. One session of CE at 50% peak oxygen consumption (VO_{2peak}; a measure of aerobic exercise capacity) for 1.5 hr reduced VLDL-TG secretion during both the exercise and recovery periods compared to pre-exercise baseline (122); however, this study lacked a sedentary control trial, so findings could not be conclusive. VLDL-TG kinetics likely vary with time since the prior meal, and so it is difficult to interpret post-exercise data in the absence of time-matched sedentary controls. Because plasma TG levels as well as expression and activity of hepatic MTP, which is involved in assembly and secretion of VLDL-TG, can vary within a 24 hr period (105), it is possible that the apparent change in secretion after

CE (122) could have been circadian in nature rather than a response to exercise. Exercise at 60% VO_{2peak} for 2 hr in men reduced secretion rate of the VLDL associated apolipoprotein B-100 but not TG compared to a sedentary control trial, suggesting a possible but unconfirmed decrease in secretion rate of VLDL particles (92). However, when subjects exercised at 60% VO_{2peak} for only 1 hr, secretion rate of apolipoprotein B-100 and TG were unchanged (90), indicating importance of exercise volume and/or energy expenditure. Although reductions in VLDL-TG secretion in men in response to single bouts of exercise have not been observed (90, 92), a reduction in VLDL-TG secretion was demonstrated in women performing a single session of exercise at 60% VO_{2peak} for 2 hr versus a sedentary control trial (9).

There is some evidence in both mice (60) and humans (32) that hepatic TG concentration is transiently elevated in response to acute CE. Hepatic TG content is increased after 3 hr but not immediately after exercise (60), indicating that this response is a post-exercise recovery phenomenon. As well, muscle TG is reduced immediately after exercise and tends to remain lower 3 hours later (60), demonstrating that the post-exercise rise in intracellular TG is tissue-specific. The physiological relevance of this rise in hepatic TG is unclear. Reductions in hepatic TG with chronic exercise are thought to correlate to improved insulin sensitivity. Temporary rises in hepatic TG after acute exercise, however, might also be beneficial. Acute bouts of CE prevent FA-induced whole-body insulin resistance partially by promoting storage of FAs as biologically inert TG (114). It is possible that a similar response also occurs in the liver.

Potential effects of exercise intensity on metabolism of hepatic TG and VLDL-TG

AMP-activated protein kinase (AMPK) is activated by stresses, such as exercise, that increase the ratio of AMP to ATP (109). In response to a rise in AMP/ATP, AMPK activity is increased by phosphorylation of Thr^{172} of the catalytic α-subunit (109). In rats, single sessions of CE are capable of enhancing AMPK activity in the liver (20, 106). This increase in AMPK activity could lead to reduced ACC and increased malonyl-CoA decarboxylase (MCD) activities, and as a result, a decrease in malonyl-CoA content (106). Interestingly, this pattern of changes in AMPK, ACC, and MCD activities, as well as malonyl-CoA concentration, occurs in liver, muscle, and adipose in response to a single exercise bout (106). As well, AMPK can inhibit sn-glycerol-3-phosphate acyltransferase, which is an early step in TG synthesis (109). In theory, the net effect would be a shift of FAs toward oxidation and away from esterification into TG, as outlined in Figure 1-1. Because hepatic AMPK and ACC are activated and deactivated, respectively, to greater extents by single bouts of high- compared to low-intensity exercise (20), the repeated bursts of intense exertion may make HIIE more effective than CE for altering hepatic lipid metabolism.

Figure 1-1

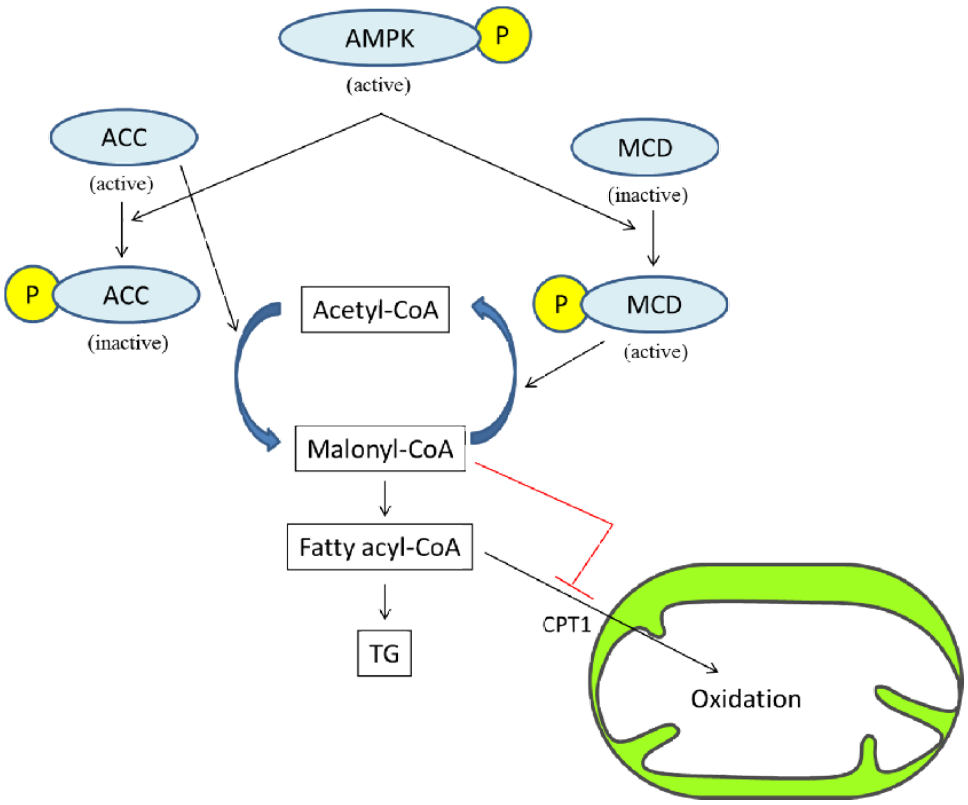

Figure 1-1. AMPK signaling and lipid metabolism. In the de-phoshorylated state, ACC is active and catalyzes the formation of malonyl-CoA from acetyl-CoA, and MCD which catalyzes the reverse reaction, is inactive. ACC and MCD are deactivated and activated, respectively, through phosphorylation by active (phosphorylated) AMPK, leading to a decrease in malonyl-CoA concentration (109). As a result, inhibition of transport of fatty acyl-CoAs via CPT1 into the mitochondria for oxidation is relieved (109), which can potentially partition fatty acyl-CoAs towards oxidation and away from TG synthesis. AMPK, AMP-activated protein kinase; P, phosphorylated; ACC, acetyl-CoA carboxylase; MCD, malonyl-CoA decarboxylase; CPT1, carnitine palmitoyl transferase-1; TG, triglyceride.

In humans, acute exercise studies suggest that there is an energy expenditure threshold required during exercise to elicit changes in VLDL-TG kinetics (86). In men, for instance, it has been shown that a single bout of CE at 60% $VO2_{peak}$ for 2 hours (92), but not 1 hour (90), enhances VLDL-TG clearance. But, when a bout of low energy expenditure, low-intensity continuous exercise (90 min at 30% $VO2_{peak}$) was compared to an energy-matched bout of high-intensity resistance exercise, only the resistance bout was capable of increasing VLDL-TG clearance (91), suggesting that intense exercise can lower the energy expenditure needed for altering the clearance aspect of VLDL-TG kinetics in men. Similarly, there appears to be an energy expenditure threshold in women, as exercise at 60% $VO2_{peak}$ for 1 hour has no impact on VLDL-TG kinetics (89) but both enhanced clearance and reduced secretion occur with 2 hours of exercise at a similar intensity (9). Whether exercise at high intensities, such as with HIIE, can lower the energy expenditure threshold to alter VLDL-TG kinetics in females remains unknown, since findings in humans regarding HIIE and VLDL-TG metabolism have only been reported for men (8, 137).

Sexual dimorphisms in lipid metabolism at rest and in response to exercise

There are many sex-based differences in lipid metabolism in the basal state and in response to CE. Women have greater concentrations of intramuscular TG (IMTG) along with greater abundance of enzymes involved in lipid oxidation (133), and utilize IMTG to a larger extent than men during CE (111). During CE, women oxidize relatively

more lipid; this could be related to the observed greater content of lipid oxidation enzymes (133) as well greater sensitivity to catecholamine-induced whole-body lipolysis (115). Although various sex differences in lipid metabolism have been demonstrated for muscle, it remains unknown whether these differences also apply to the liver. It is possible that hepatic lipid is also utilized to a greater degree in women during exercise.

In the post-exercise period, men exhibit greater elevations in total energy expenditure, whole-body lipolysis, and fat oxidation than women (52). As well, postprandial lipemia in response to exercise could possibly be attenuated to a larger extent in men than women (52). With regard to VLDL-TG secretion, it has been demonstrated that women exhibit a higher secretion rate under basal sedentary conditions (88, 98). However, molecular mechanisms underlying this sexual dimorphism are unknown and an animal model is needed to study this phenomenon. Whether sex differences in VLDL-TG secretion rate in response to exercise exist has not been established. Although VLDL-TG secretion rate was not reduced by a single recent bout of CE in men (90, 92) , for a study conducted in women performing a single session of exercise at 60% VO_{2peak} for 2 hr versus a sedentary trial, a significant reduction in VLDL-TG secretion was reported (9), revealing potential sex differences in this response. However, direct comparisons of males vs. females are needed to conclusively demonstrate sexual dimorphisms in VLDL-TG secretion in response to exercise.

Exercise and Perilipin 2

Another mechanism through which exercise could influence hepatic TG metabolism and secretion is through changes in LD-associated protein content. Perilipin 2 (PLIN2, also known as adipose differentiation-related protein and adipophilin) is a LD-associated protein in the liver (131, 148) and other tissues (15) and is the primary perilipin isoform that is associated with LDs in liver (148). PLIN2 plays a major role in hepatic TG metabolism, as evidenced by up to 60% reductions in hepatic TG content with PLIN2 knock-down (21, 22, 62). As well, in some (22, 72) but not all (62) studies, knock-down of PLIN2 results in higher plasma TG concentrations in the fasted state during administration of an inhibitor of plasma TG degradation, suggesting a possible but unconfirmed increase in rates of VLDL-TG secretion (51). As expected based on loss-of-function studies, the opposite effect on hepatic TG and secretion is observed when PLIN2 is overexpressed. In cultured rat hepatocytes transfected with PLIN2, hepatic TG concentration is increased and VLDL-TG secretion decreased (93), further demonstrating the involvement of PLIN2 in hepatic TG metabolism and VLDL-TG secretion.

Chronic CE increases both muscle PLIN2 and IMTG (119, 120). Importantly, chronic HIIE produces similar increases in resting IMTG and muscle PLIN2 protein as CE even though less work is performed (120). As certain alterations in lipid metabolism in response to exercise occur in both muscle and liver (106), it is possible a similar intensity-dependent phenomenon may occur in liver. Elevations in PLIN2 could promote increases in intracellular TG by preventing access of lipases to lipid droplets (14, 77), while increases in hepatic TG could possibly elevate PLIN2 protein abundance

through protein stabilization (94, 152). Exercise leads to increased plasma FFAs due to adipose tissue lipolysis thereby potentially increasing FA availability for hepatic TG synthesis. Furthermore, FAs are ligands for peroxisome proliferator-activated receptor-α (PPARα) (38), which is a known transcriptional activator of PLIN2 (27, 31), and indeed exercise stimulates hepatic PPARα expression (65). Thus, it is plausible that exercise can increase hepatic TG content by both increased FA availability for TG synthesis as well as stabilization of lipid droplets by PLIN2. As well, PLIN2 protein could possibly be increased in response to exercise via protein stabilization by increased lipid droplet content and enhanced gene transcription. Despite the emerging work studying the relationship between PLIN2 and TG metabolism in muscle with exercise, however, little is known in this regard with liver and it may be that some of the potential benefits of exercise on hepatic lipid metabolism could be mediated by this LD-associated protein.

Metabolic impairments with menopause

Menopause is defined as the permanent cessation of menstruation confirmed by 12 consecutive months without a period (1). Menopause leads to impairments in lipid metabolism as evidenced by higher fasting plasma TG (10, 28, 43, 95, 145) and VLDL-TG secretion rate (87) as well as fatty liver (45-47). In addition, menopause is also associated with other metabolic and neuromuscular impairments including insulin resistance (74, 85), increased fat mass (40, 58, 70, 81, 85, 129, 134), and loss of muscular strength (67). Controlled studies in postmenopausal women show CE to be

capable of lowering blood TG (75) as well as modestly reducing body weight (3), body fat (3), and blood glucose (2), but the effects of exercise intensity on these parameters are unknown.

The ovariectomized mouse is an animal model of menopause that recapitulates many of the impairments that post-menopausal women experience (42, 102, 112, 125, 155) and thus can be used for preclinical study of novel intervention strategies, such as HIIE. Ovariectomized mice display increases in fat mass (112), impaired insulin sensitivity (125), and decreases in muscular strength (102). The effect of ovariectomy in mice on hepatic TG however, is less understood as some studies have shown it to be elevated (42, 112, 155) or unchanged (63). These inconsistent findings may be related to type of diet (82) and fasting duration prior to tissue collection (34, 103). Although ovariectomy may not always lead to increased hepatic TG content, it appears however to increase susceptibility to high fat diet-induced hepatic steatosis (82).

Mouse models of HIIE

Although much work is emerging studying HIIE in humans (6, 7, 44, 78-80, 120, 135, 136), there are only a small number of studies involving HIIE in mice and these studies focused on effects on heart (64, 124) and muscle (64) function and hypertrophy. In these mouse studies, HIIE consisted of alternating between high-intensity intervals at 85-90% VO_{2max} of 4-8 minutes and low-intensity periods at 50-60%VO_{2max} for 2 minutes for a total of 80 min to 2 hr on a 25 degree inclined treadmill. Because there is no gold

standard on how to apply HIIE and the potential intensity-dependent effects on hepatic lipid metabolism (20), we performed some unpublished development work to determine a challenging interval exercise protocol for mice, titrating intensity and exercise/rest intervals to exclude protocols that were too difficult (i.e., most mice could not complete it) and to work our way back to an intensity that appears to be the most challenging protocol that essentially all mice could complete. The following protocols were ultimately selected and were used for Specific Aims 1 and 2 (Figure 1-2). For HIIE, following a 5 minute warmup at a slow walking speed (5 m/min), mice ran for 30 second running intervals with 60 second walking rest periods (5 m/min) interspersed between intervals. The first, second, and third running intervals were at 15, 20, and 25 m/min, respectively, followed by all remaining sprint intervals at a final speed of 30 m/min. Both the warmup and the exercise session were performed at an incline of 25°. CE consisted of an incline-matched, duration-matched, and distance-matched continuous running session (13.8 m/min for 30 min) following the same 5 minute warmup phase. In our preliminary HIIE protocol development, we observed that mice of the age and strain used in Specific Aims 1 and 2 with the treadmill at a 25 degree incline could run at 30m/min for only a short period of time (approximately 30s) before becoming fatigued. Thus, our HIIE protocol (Figure 1-2) likely achieves much higher relative intensities during exercise than previously published work (64, 124). As well, the total duration of our exercise bouts was less than half of those used previously (30-45 minutes vs. 80 min to 2 hr), which was needed for the mice to tolerate higher intensity of exercise.

Figure 1-2

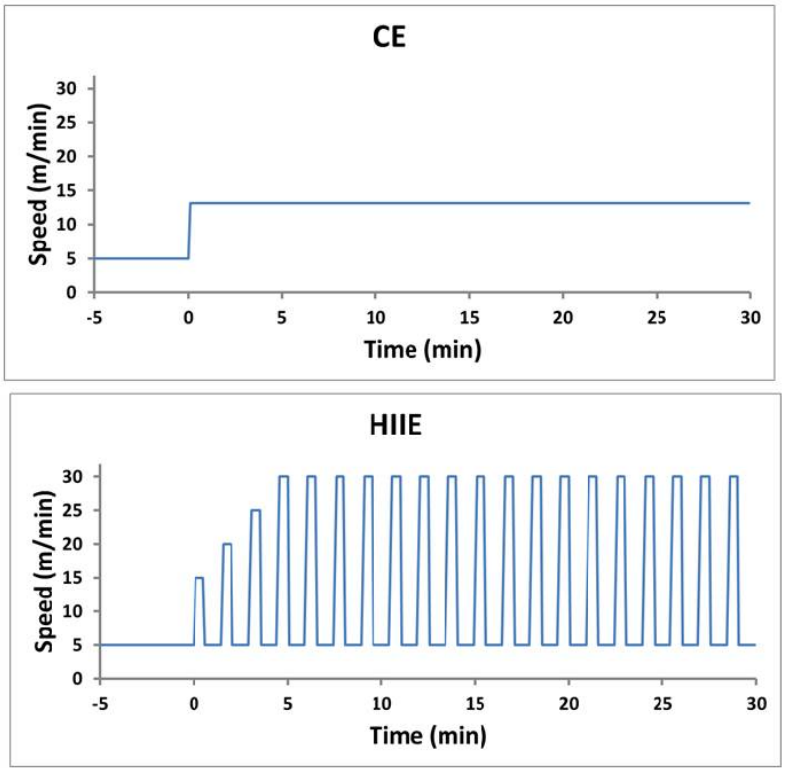

Figure 1-2. Final exercise protocols after method development. Following a 5 min warmup at 5 m/min, exercise sessions for CE and HIIE were 30 min in duration in Specific Aim 1 and 30-45 min in duration for Specific Aim 2. For HIIE, mice ran for 30 s sprint intervals interspersed with 60 s walking rest periods at 5 m/min. The first, second, and third sprint intervals were at 15, 20, and 25 m/min, respectively, followed by all remaining sprint intervals at 30 m/min. Acceleration to final sprint interval speeds was performed within 5 s and deceleration back to 5 m/min within 2 s. CE bouts were distance-, duration-, and incline- matched runs at a constant speed of 13.8 m/min. CE and HIIE were performed at a 25° incline and matched for total distance ran. Energy expenditure during these bouts was assessed in Specific Aim 3.

VLDL-TG secretion rate measurement in rodents

Assessment of hepatic VLDL-TG secretion rate in rodents can be performed by blockade of circulating TG degradation in the fasted state by administration of tyloxapol, which blocks intravascular lipases such as lipoprotein lipase and hepatic lipase (51). This approach produces values similar to experiments using radioactive tracer (11). After administration of tyloxapol, complete blockade of blood TG degration is achieved and a linear rise in blood TG concentration over time occurs (Figure 1-3), with the slope of the regression indicating hepatic TG secretion rate (51). Because there is a variable lag phase until complete blockade is reached [(71) and unpublished data from our lab], it is ideal to identify the range of the linear increase in blood TG concentration of each individual mouse for accurate calculation of secretion rate (51).

Figure 1-3

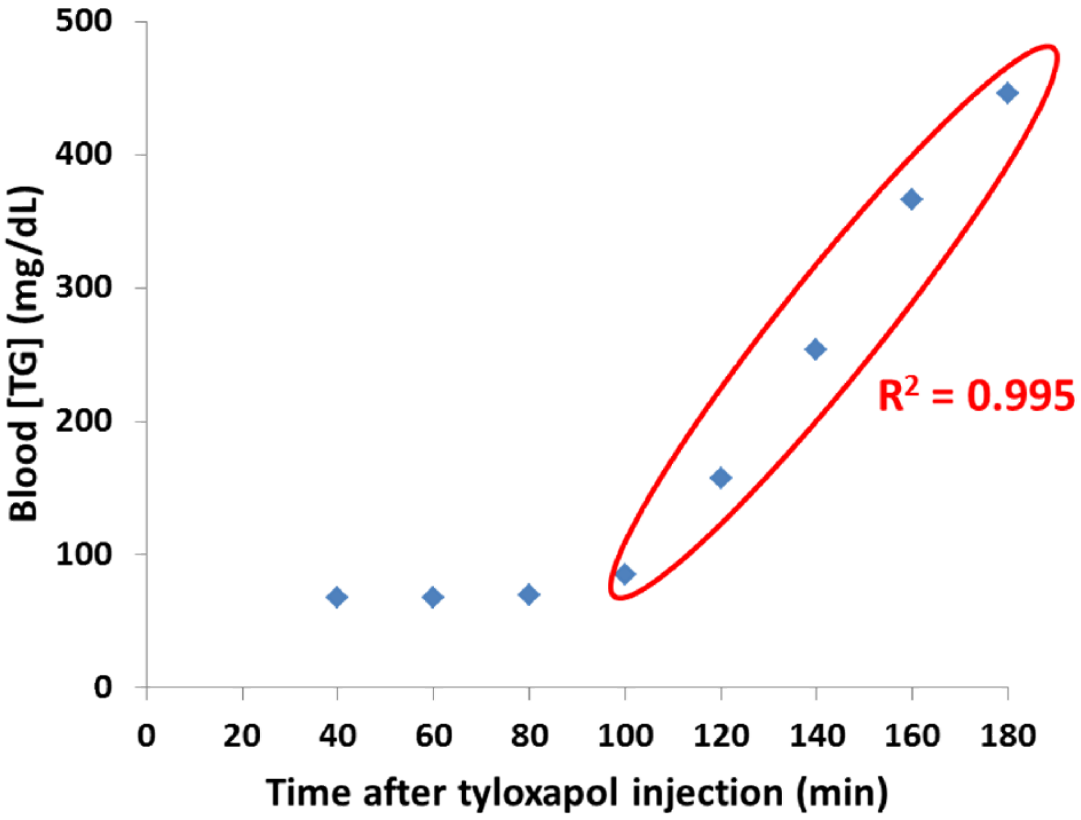

Figure 1-3. Representative trace of blood TG concentration after intraperitoneal injection of tyloxapol (500 mg/kg BW) from an individual fasted mouse used in Specific Aim 1. After a lag phase, complete blockade of blood TG degradation is reached followed by a linear increase in concentration of blood TG (encircled). The slope of the linear regression line indicates the hepatic VLDL-TG secretion rate (51). A high degree of linearity (R^2~0.99) was consistently achieved.

Energy substrate oxidation and exercise intensity

The respiratory exchange ratio (RER) is the ratio of carbon dioxide (VCO_2) produced to oxygen consumed (VO_2) as measured from the mouth. RER is used as an indicator of relative substrate (lipid vs. carbohydrate) oxidation within the cell based on the following stoichiometric relationships (59) :

$$C_6H_{12}O_6 \text{ (glucose)} + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$$

$$C_{16}H_{32}O_2 \text{ (palmtitic acid)} + 23\ O_2 \rightarrow 16\ CO_2 + 16\ H_2O$$

RER for complete oxidation of glucose is 6 CO_2 /6 O_2 = 1 and 16 CO_2 /23 O_2 = 0.7 for palmitic acid (an abundant FA used for fuel). RER determines the caloric equivalence of VO_2, and together they can be used to calculate true metabolic rate (kcal per unit time) (52). Thus, measurement of gas exchange can be a valuable tool for assessing both energy expenditure and relative contribution of fuels. At rest, lipid oxidation provides the greatest proportion of total energy. During CE the relative contribution of lipid declines and carbohydrate rises as the relative intensity of exercise in increased, and at approximately 75% of VO_{2peak}, carbohydrate becomes the dominant substrate for fuel oxidation [Figure 1-4, (17)]. Thus, RER approaches 1 as relative intensity of exercise is increased and declines towards 0.70 as it is decreased. Through training (habitual exercise) however, the absolute intensity of exercise (e.g., a given speed on a treadmill or cycle ergometer) after compared to before training is relatively less intense (a lower percentage of VO_{2peak}) (17).

Figure 1-4

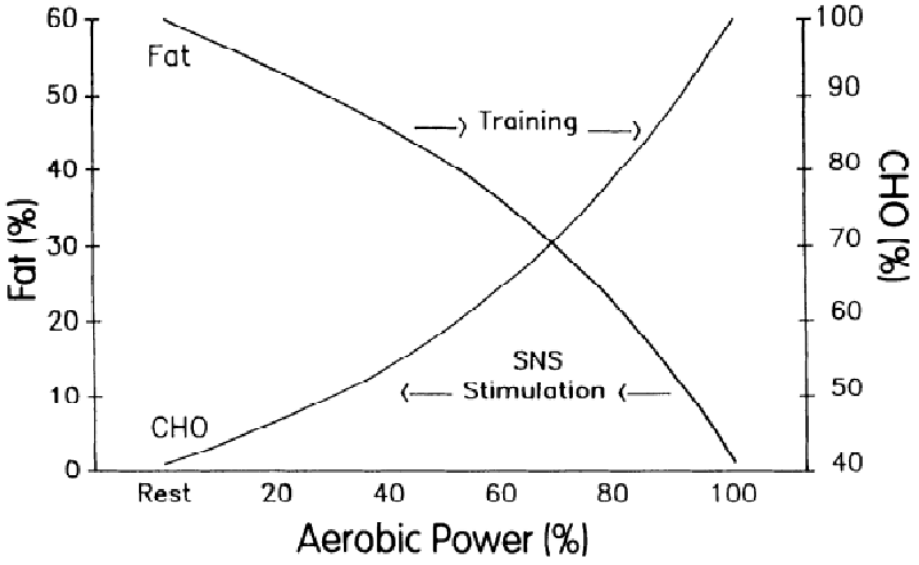

Figure 1-4. Relative increase in energy derived from carbohydrate (CHO) utilization and decline in energy from oxidation of lipid (fat) utilization as function of relative power output. At crossover point, increments in relative exercise intensity result in increasingly greater dependence on CHO and less dependence on fat. Even though on absolute scale training results in rightward curve shifts, on relative basis training probably has minimal effects on curves relative to aerobic power. SNS, sympathetic nervous system.

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Specific Aims

Elevated hepatic TG concentration is associated with metabolic impairments such as insulin resistance (33, 66, 118) and increased VLDL-TG secretion (33). Because of these negative health effects, interventions are needed that favorably alter hepatic TG metabolism. Exercise is an attractive approach, but the relative effectiveness of exercise modalities of different intensity on hepatic TG metabolism is largely unknown. As well, the distinct responses in hepatic TG metabolism to acute vs. chronic exercise are not entirely clear. To address these gaps in knowledge, we combined molecular evaluation of hepatic TG metabolism and hepatic TG measurement along with kinetic assessment of VLDL-TG secretion in mice following acute and chronic exposure to CE and HIIE.

Specific aim 1: To determine the impacts of single bouts of HIIE vs. CE on hepatic TG metabolism and secretion.

Cumulative effects of single exercise bouts likely play a major role in the benefits of chronic exercise training (15, 24, 25) and could possibly be enhanced when exercise is performed at high intensities. Therefore, the purpose of this aim was to assess the influence of one session of HIIE vs. CE on hepatic TG metabolism and secretion as well as to determine molecular mechanisms underlying these physiological results. VLDL-TG secretion rates, hepatic TG content, and abundance and surrogate markers of activation for key hepatic proteins involved in TG metabolism and secretion were measured in

sedentary controls and mice that performed one session of either HIIE or CE, matched for work load and bout duration. Because of sex-based differences in exercise metabolism (62) and basal VLDL kinetics (37, 44), we assessed the response to these exercise modalities in both sexes. We hypothesized that VLDL-TG secretion rate would be reduced in association with lower hepatic TG concentration and enhanced AMPK signaling following HIIE but to a lesser extent with CE and that exercise effects would be more pronounced in females.

Specific aim 2: To determine the impacts of chronic HIIE vs. CE on hepatic TG metabolism and secretion, body composition, glucose tolerance, and strength in ovariectomized and sham-operated mice.

The ovariectomized mouse is an animal model of menopause that recapitulates many of the impairments that post-menopausal women experience (42, 102, 112, 125, 155) and can be used for preclinical study of novel intervention strategies. Because of the potential for exercise training to reduce severity of complications associated with menopause, we compared 6 weeks of training using these exercise protocols on hepatic TG metabolism and secretion, glucose tolerance, body composition, and grip strength in ovariectomized (OVX) and sham-operated (SHAM) mice. We hypothesized that HIIE would attenuate changes associated with ovarian hormone deficiency to a greater extent than CE.

Specific aim 3: To characterize HIIE and CE in terms of substrate oxidation and metabolic rate during and after exercise as well as post-exercise spontaneous physical activity.

In this aim, energy expenditure and substrate oxidation both during and immediately after exercise, as well as post-exercise spontaneous physical activity (SPA), were assessed in a subset of mice used in Specific Aim 2 to further characterize metabolic and behavioral responses to these exercise modalities. Indirect calorimetry was performed on OVX and SHAM mice during exercise (or time-matched sedentary condition) in an enclosed treadmill. For assessment during the post-exercise recovery period, OVX and SHAM mice were placed into an indirect calorimetry/activity monitoring system for 4 hours immediately after exercise (or time-matched sedentary condition). Because of the intense nature of HIIE, we hypothesized that relative lipid oxidation would be lower during and higher after exercise in HIIE vs. CE. We also hypothesized that SPA would be reduced following exercise to compensate for the activity of exercise.

Chapter 2: Intensity-dependent and sex-specific alterations in hepatic triglyceride metabolism in mice following acute exercise

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Journal of Applied Physiology (2015) 118, 61-70

Author contributions:

M.A.T. and G.C.H. conceived of and designed the research; T.G.A. contributed to design of research; M.A.T. performed experiments with assistance from T.R.M., G.J.W., and G.C.H.; M.A.T. analyzed data; M.A.T. and G.C.H. interpreted results of experiments; M.A.T. prepared figures; M.A.T. and G.C.H. drafted the manuscript; M.A.T., T.R.M., G.J.W., T.G.A., and G.C.H. edited and revised the manuscript; M.A.T., T.R.M., G.J.W., T.G.A., and G.C.H. approved the final version of the manuscript.

Abstract

Precise regulation of hepatic triglyceride (TG) metabolism and secretion is critical for health and exercise could play a significant role. We compared one session of high-intensity interval exercise (HIIE) vs. continuous exercise (CE) on hepatic TG metabolism. Female and male mice were assigned to CE, HIIE, or sedentary control (CON). HIIE was a 30min session of 30s running intervals (30m/min) interspersed with 60s walking periods (5m/min). CE was a distance- and duration-matched run at 13.8m/min. Hepatic content of TG and TG secretion rates, as well as expression of relevant genes/proteins, were measured at 3 hr (Day 1) and 28 hr (Day 2) post-exercise. On Day 1, hepatic [TG] in CE and HIIE were both elevated vs. CON in both sexes with ~2-fold greater elevation in HIIE vs. CE in females. In both sexes, hepatic perilipin 2 (PLIN2) protein on Day 1 was increased significantly by both exercise types with a significantly greater increase with HIIE than CE, while the increase in mRNA reached significance only after HIIE. On Day 2 in both sexes the increases in hepatic TG and PLIN2 with exercise declined toward CON levels. Only HIIE on Day 2 resulted in reduced hepatic TG secretion by ~20% in females with no effect in males. Neither exercise modality altered AMPK signaling or microsomal triglyceride transfer protein expression. Females exhibited higher hepatic TG secretion than males in association with different expression levels of related metabolic enzymes. These intensity-dependent and sex-specific alterations following exercise may have implications for sex-based exercise prescription.

Keywords: Physical activity, lipoprotein, gender, HIIT, HIT, postexercise recovery, post-exercise

Introduction

Dysregulated hepatic lipid metabolism is linked with development of chronic disease. Elevated plasma triglyceride (TG) concentrations, circulating in the fasted state primarily as very-low density lipoprotein-TG (VLDL-TG), and excess hepatic TG accumulation are associated with increased coronary heart disease risk (4) and insulin resistance (33, 66, 118), respectively. Both plasma TG (30) and hepatic TG content (128, 149) decrease with chronic endurance exercise. However, much of the effect of chronic exercise on TG metabolism is likely due to acute impacts of repeated single exercise bouts rather than training-induced adaptations per se (49, 56). There was some initial evidence in a human study that single bouts of moderate-intensity continuous exercise (CE) may reduce the rate of VLDL-TG secretion compared to pre-exercise baseline (122); however, this study lacked a sedentary control trial, so findings could not be conclusive. VLDL-TG kinetics and other aspects of hepatic TG metabolism likely vary with time since the prior meal, and so it is difficult to interpret post-exercise data in the absence of time-matched sedentary controls. Because plasma TG levels as well as expression and activity of hepatic microsomal triglyceride transfer protein (MTP), which is involved in assembly and secretion of VLDL-TG, can vary within a 24 hr period (105), it is possible that the apparent change in secretion after CE (122) could have been circadian in nature

rather than a response to exercise, further emphasizing the necessity of comparing changes with exercise to time-matched sedentary controls as opposed to pre-exercise baseline. Although VLDL-TG secretion rate was not reduced by a recent bout of CE in men compared to a sedentary trial (92), for a study conducted in women performing an exercise session of similar duration and relative intensity versus a sedentary trial, a significant reduction in VLDL-TG secretion was reported (9), revealing this aspect of lipid metabolism can be altered by acute exercise with possible sex differences in this response. Additionally, it has been demonstrated that women exhibit a higher VLDL-TG secretion rate under basal sedentary conditions (88, 98), and an animal model of this phenomenon is needed to create opportunity to understand the molecular mechanisms responsible for the sexual dimorphism observed.

High-intensity interval exercise (HIIE) involves alternating between relatively easy and challenging intensities within a single exercise bout and may be a more effective means of improving hepatic lipid metabolism than the traditional endurance exercise approach of CE at a steady workload because of the potential metabolic effects of the short periods of high intensity exertion. Chronic (137) but not acute (8) HIIE has been shown to lower VLDL-TG secretion rate in men. However, the effects of HIIE on VLDL-TG secretion in female participants have yet to be reported and the effectiveness of HIIE vs. CE needs to be directly compared. As well, molecular mechanisms underlying changes in VLDL-TG secretion and other aspects of hepatic lipid metabolism by exercise are poorly understood.

It is plausible that one session of HIIE could lower VLDL-TG secretion rate to a greater degree than CE through modulation of hepatic AMP-activated protein kinase (AMPK) activity. In rats, a single exercise session of CE was sufficient to increase activity of AMPK in the liver (20, 106), but it was not known if HIIE could have a more robust effect upon this pathway. This increase in AMPK activity could lead to reduced acetyl-CoA carboxylase (ACC) and increased malonyl-CoA decarboxylase activities, and as a result a decrease in malonyl-CoA content (106). In theory, the net effect would be a shift of fatty acids (FAs) toward oxidation and away from esterification into TG. Since hepatic AMPK and ACC are activated and deactivated, respectively, to greater extents by single bouts of high- compared to low-intensity exercise (20), HIIE is potentially more effective than CE for altering hepatic lipid metabolism because of the repeated bursts of highly intense activity.

In addition to potential effects on AMPK signaling, another route by which exercise might alter hepatic TG metabolism and secretion is through changes in lipid droplet (LD)-associated protein content. Perilipin 2 (PLIN2) is a LD-associated protein in the liver (131, 148) and other tissues (15) and is the primary perilipin isoform that is strictly associated with LDs in liver (148). PLIN2 may inhibit intracellular TG lipolysis by blocking access of LDs to cytosolic lipases (14) and thus changes in its expression can possibly alter intracellular TG abundance and FA trafficking (93). Indeed, loss of function of PLIN2 in mice results in 25-60% reductions in hepatic TG content (21, 22, 62) and involvement of PLIN2 in TG secretion has been demonstrated in PLIN2 transfected hepatocytes (93), revealing an important role of this protein in hepatic TG metabolism.

There is some evidence in mice that acute CE transiently raises hepatic TG in the post-exercise recovery period compared to time-matched sedentary controls (60), however, the role of PLIN2 and the influence of exercise intensity in this phenomenon is unknown.

The effects of single exercise bouts likely play a major role in the benefits of chronic exercise training (29, 53, 55) and could possibly be enhanced when exercise is performed at high intensities, thus highlighting the importance of assessing the acute impact of different exercise modalities. Therefore, the purpose of this study was to assess the influence of one session of HIIE vs. CE on hepatic TG metabolism and secretion as well as to determine molecular mechanisms underlying these physiological results. VLDL-TG secretion rates, hepatic TG content, and abundance and surrogate markers of activation for key hepatic proteins involved in TG metabolism and secretion were measured in mice that performed one session of either HIIE or CE, matched for work and bout duration. Because of the many known sex-based differences in exercise metabolism (133) as well as the known sex differences in basal VLDL kinetics in humans (88, 98), our purpose was also to determine sex differences in these responses. Furthermore, we included time-matched sedentary groups to control for potential diurnal variations in hepatic lipid metabolism as well as effects of the time-elapsed since food withdrawal. We hypothesized that VLDL-TG secretion rate would be reduced in association with lower hepatic TG concentration and enhanced AMPK signaling following HIIE but to a lesser extent with CE and that exercise effects would be more pronounced in female mice.

Methods

Animals. This protocol was approved by the Rutgers University Institutional Animal Care and Use Committee. Male and female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were maintained on a 12-hour light/dark photoperiod with all mice allowed ad libitum access to food and water. All mice consumed the Labdiet 5K52 diet (Purina Mills, Richmond, IN) for their lifetimes and were acclimated to the facility for at least 5 days prior to exercise. Body composition was assessed between 14-16 weeks of age, prior to exercise, using an EchoMRI system (Echo Medical Systems, Houston, TX).

Exercise protocols. Mice were exercised between the ages of 14-16 weeks on a treadmill (Exer-3/6, Columbus Instruments, Columbus, OH) with a shock grid set at a low intensity (on a scale of 0-10, set at 1). On the day before exercise, mice were acclimated to the treadmill for 5 minutes at a speed of 5 m/min with no incline (0°). At 7:00AM on the day of exercise food was withdrawn and at 10:00AM a drop (approximately 15 µL) of whole blood was drawn from the tail for measurement of pre-exercise TG concentration using a hand-held meter (Cardiochek, Polymer Technology Systems, Inc., Indianapolis, IN). Mice were then assigned to time-matched sedentary control (CON), CE, or HIIE groups matched for pre-exercise TG concentration followed by exercise beginning between 11:45AM and 12:30PM of the same day. CE and HIIE are described below, and CON was a time-of-day matched condition in which mice remained in their cages with water bottles withdrawn for the amount of time that CE and HIIE mice would spend away from their cages during a session (approximately 35 minutes). Effects of CE and

HIIE were compared only to CON within the same day to control for drift in sedentary baseline over time.

Preliminary development work was performed to establish an HIIE protocol that would be challenging but that could be consistently completed, and ultimately the protocol reported here was chosen. For HIIE, following a 5 minute warmup at a slow walking speed (5 m/min), mice ran for 30 second intervals with 60 second walking rest periods (5 m/min) interspersed between intervals. The exercise session included 20 running intervals, the first at 15 m/min, next at 20 m/min, then at 25 m/min, followed by all remaining sprint intervals at a final speed of 30 m/min. Acceleration up to 15, 20, 25 and 30 m/min was performed within a 5 second ramping duration and deceleration back to 5 m/min within a 2 second duration. Both the warmup and the exercise session were performed at an incline of 25°. CE consisted of an incline-matched, duration-matched, and distance-matched continuous running session (13.8 m/min for 30 min) following the same 5 minute warmup phase. To avoid repeated shocks and to maintain running speed, mice were gently prodded by hand when they closely approached the shock grid. As described below, measurements and tissue collections were made either on the day of exercise (Day 1) or on the following day (Day 2).

Food intake. Overnight food intake was measured in mice that were assigned to be killed on Day 2. In these mice, at 7:00PM on the day of exercise, a known amount of food was added back to cages and the remaining food at 7:00AM the following morning was weighed.

Hepatic TG secretion. Food was withdrawn at 7:00AM on the day of experimentation. Blood TG rate of appearance (Ra), considered to represent the hepatic VLDL-TG secretion rate, was assessed at 3:00PM following an 8-h fast on the day of exercise (Day 1) and the day following exercise (Day 2) in separate sets of mice, with a minimum of 6 mice per group. Blood TG was measured on samples (approximately 15 μL) obtained from the tail after i*ntraperitoneal* injection of tyloxapol (500 mg/kg) (Sigma-Aldrich, St. Louis, MO) taken at 20-min intervals until TG exceeded 500 mg/dL, as described and discussed previously (51), with the same handheld meter used to measure pre-exercise TG concentration. Tyloxapol prevents circulating TG degradation such that blood TG concentration rises linearly, the slope of the linear regression indicating the rate of VLDL-TG secretion (51). A high degree of linearity ($R^2 \approx 0.99$) was consistently achieved. Hepatic VLDL-TG secretion rate was measured per unit of blood volume (mg/dL/min) and then converted to values normalized to body weight (mg/kg BW/min) based upon an assumption of blood volume (116).

Tissue collection. Tissue collection was performed in a separate set of mice than those used for measurement of VLDL-TG kinetics with a minimum of 6 mice per group. Again, food was withdrawn at 7:00AM and then tissues were collected at the same time-of-day as the hepatic TG secretion measurements described above. On the day of exercise (Day 1) or the day after exercise (Day 2), mice were euthanized by CO_2 inhalation followed by immediate collection of blood via cardiac puncture. Blood was collected in EDTA-coated tubes followed by isolation and storage of plasma at -80°C until analysis.

Liver tissues were quickly collected following blood draw, immediately frozen in liquid nitrogen then stored at -80°C until analysis.

Biochemical assays. Plasma glycerol (a marker of whole body lipolysis) and plasma TG concentrations were measured using a commercially available kit (Sigma-Aldrich). For measurement of hepatic TG concentration, lipids were extracted from approximately 8 mg of tissue and TG content of the extract was determined using a commercially available kit (Sigma-Aldrich) (37, 54) and expressed as percentage of liver wet weight.

Western blotting. Western blotting was conducted as previously reported by our group (54) with minor modification. Briefly, approximately 40 mg of liver was homogenized followed by gel electrophoresis of 50 μg protein and transfer onto membranes. Membranes were incubated with primary antibodies against total AMPKα (1:400; Cell Signaling Technology, Danvers, MA), phospho-AMPKα^{Thr172} (1:800; Cell Signaling Technology), total ACC (1:8,000; Abcam, Cambridge, MA), phospho-ACC^{Ser79} (1:1,000; Cell Signaling Technology), PLIN2 (1:25,000; kindly provided by Dr. Dawn Brasaemle of Rutgers University), MTP (1:100,000; BD Transduction Laboratories, San Jose, CA), cAMP response-element binding protein (CREB, 1:500; Cell Signaling Technology), phospho-CREB^{Ser133} (1:1,000; Cell Signaling Technology) and β-actin (1:2,000; Cell Signaling Technology). Membranes were then incubated with IR Dye 680 (1:10,000; LI-COR Biosciences, Lincoln, NE) or IR Dye 800 (1:10,000; LI-COR Biosciences) secondary antibodies and bands quantified with β-actin as a loading control (54).

mRNA quantitation. Approximately 20 mg of liver was pulverized under liquid nitrogen temperature and RNA was isolated (18) followed by treatment with RNAsecure (Life Technologies, Grand Island, NY) and DNase 1 (Life Technologies). cDNA synthesis was performed using a commercially available kit (Life Technologies) and RT-PCR using the following TaqMan Gene Expression Assays (Life Technologies): PLIN2 (Assay ID Mm00475794_m1), AMPKα1 (Assay ID Mm01296700_m1), ACC1 (Assay ID Mm01304257_m1), ACC2 (Assay ID Mm0120467_m1), MTP (Assay ID Mm00435015_m1) and 18s ribosomal RNA (cat. no. 4352930E) as the endogenous control as detailed previously (18) and results are expressed as fold-difference relative to female sedentary control mice.

Statistical analysis.

Data are presented as means ± SE. Responses to exercise (trial) were analyzed by 2-way ANOVA (sex-by-trial) within each day. As discussed above regarding the importance of controlling for drift in sedentary baseline over time, effects of exercise were only compared to CON of the same day. For determination of sex differences under sedentary conditions, ANCOVA examining the effect of sex across both Days 1 and 2 (Day as the covariate) was used in order to collectively analyze these data to test for general sex differences in the control condition. To directly probe differences between CE and HIIE, a priori planned comparisons by t-test were conducted to compare relative changes from CON between these two conditions. ANOVA was followed by Fisher's Least Significant Difference (LSD) *post hoc* test. Pearson's correlation coefficient was

used to quantify the relationships between hepatic TG concentration and VLDL-TG secretion and between PLIN2 mRNA and protein abundances in response to exercise. Statistical analyses were performed with JMP version 10 (SAS Institute Inc., Cary, NC) and alpha less than 0.05 was considered statistically significant.

Results

Characteristics of sedentary female and male mice

Compared to males, females in the CON group exhibited a lower plasma [TG] ($p<0.05$) and higher VLDL-TG secretion ($p<0.05$) both as measured per unit blood volume and normalized to body weight with similar patterns on Day 1 (5.54±0.22 vs. 4.86±0.41 mg/dL/min, 6.10±0.24 vs. 5.34±0.45 mg/kg BW/min) and Day 2 (5.42±0.35 vs. 4.30±0.27 mg/dL/min, 5.96±0.38 vs. 4.75±0.30 mg/kg BW/min) (Table 2-1). Prior to exercise, bodyweight ($p<0.0001$) and fat-free mass ($p<0.0001$) were lower and percent body fat higher ($p<0.05$) in females compared to males (Table 2-1).

Food intake

Overnight food intake is shown in Table 1-1. In response to CE and HIIE, food intakes were reduced compared to CON (main effect of trial, $p<0.01$; CE: -11.5±4.3% ($p<0.05$), HIIE: -10.5±2.9% ($p<0.05$)). There were no differences in the reductions in food intake between CE and HIIE and no sex-by-trial interactions.

TG kinetics

On Day 1, VLDL-TG secretion rate was not altered by exercise (Figure 2-1A). On Day 2, there was a sex-by-trial interaction for VLDL-TG secretion ($p<0.05$; Figure 2-1B). VLDL-TG secretion was decreased by approximately 20% on Day 2 with HIIE compared to CON in females ($p<0.05$), with no effect of CE, and there were no significant exercise-related changes in males. Plasma [TG] was not altered by exercise on Day 1 (Females, CON: 38±5 mg/dL, CE: 48±5 mg/dL, HIIE: 51±8 mg/dL; Males, CON: 54±5 mg/dL, CE: 62±5 mg/dL, HIIE: 55±4 mg/dL) or Day 2 (Females, CON: 41±5 mg/dL, CE: 39±5 mg/dL, HIIE: 39±5 mg/dL; Males, CON: 49±7 mg/dL, CE: 35±6 mg/dL, HIIE: 50±7 mg/dL). Plasma [glycerol] was also not altered by exercise on Day 1 (Females, CON: 0.30±0.03 mM, CE: 0.30±0.03 mM, HIIE: 0.31±0.03 mM; Males, CON: 0.30±0.02 mM, CE: 0.30±0.02 mM, HIIE: 0.31±0.02 mM) or Day 2 (Females, CON: 0.32±0.02 mM, CE: 0.41±0.04 mM, HIIE: 0.34±0.04 mM; Males, CON: 0.25±0.02 mM, CE: 0.26±0.02 mM, HIIE: 0.27±0.03 mM).

Hepatic TG and PLIN2

Hepatic TG concentration on Day 1 increased with exercise (main effect of trial, $p<0.0001$; Figure 2-2A) with post hoc testing indicating that both CE and HIIE were different from CON ($p<0.05$). In females, the relative increase in hepatic TG concentration compared to CON was approximately 2-fold greater with HIIE than with CE ($p<0.05$) with no significant difference between CE and HIIE in males. For PLIN2 protein on Day 1, there was a main effect of trial ($p<0.0001$) with post hoc testing indicating that both types of exercise increased abundance compared to CON with a greater increase with HIIE than CE ($p<0.05$; Figure 2-3A). mRNA expression followed a

similar pattern as protein for PLIN2 (main effect of trial, $p<0.05$; Figure 2-3C) but with post hoc testing revealing that only HIIE and not CE resulted in significant increases in mRNA expression ($p<0.05$). Regression analysis showed that group means for content of PLIN2 mRNA and protein were highly correlated ($R^2=0.95$, $p=0.001$). On Day 2, hepatic TG concentration (main effect of sex, $p<0.0001$; Figure 2-2B) and PLIN2 protein content (main effect of sex, $p=0.0001$; Figure 2-3B) were higher in females than males. Compared to CON within Day 2 in both sexes, TG and PLIN2 abundance in CE and HIIE were not different, indicating that the elevations found on Day 1 (Figures 2-2A and 2-3A) with both types of exercise in each sex had significantly subsided. However, there were divergent trends on Day 2 between sexes in response to HIIE (Figure 2-2B). Compared with CON, hepatic TG concentration tended to be elevated following HIIE in females ($p=0.1$) and lowered following HIIE in males ($p=0.1$). Regression analysis of group means showed that as compared to CON, changes in VLDL-TG secretion rate and changes in hepatic TG concentration on Day 2 were highly correlated inversely ($R^2=0.88$, $p<0.01$; Figure 2-2D) with no significant correlation on Day 1 (Figure 2-2C). There were no sex-by-trial interactions on Day 1 or Day 2 for hepatic TG or PLIN2 protein and mRNA.

Intracellular signaling

In sedentary mice, total ($p<0.0001$) and phosphorylated ($p=0.0001$) AMPK as well as total ($p=0.0002$) and phosphorylated ($p=0.0015$) ACC protein levels were greater in females than males. Similarly, on Day 1 and Day 2, protein levels of total and phosphorylated for both AMPK and ACC were also higher in females (main effects of

sex, $p<0.01$; Figures 2-4 and 2-5). ACC1 ($p<0.001$) and ACC2 ($p<0.05$) mRNA were higher in females on Day 2 (main effect of sex; Figure 2-5). There were no significant main effects of trial or sex-by-trial interactions on Day 1 or Day 2 for total, phosphorylated, or the ratio of phosphorylated to total protein for AMPKα and ACC (Figures 2-4 and 2-5) as well as for mRNA abundance of AMPKα1, ACC1, or ACC2 (Figures 2-4 and 2-5). The ratio of phospho-CREB^{Ser133} to total CREB (a surrogate marker of PKA activity) was greater in sedentary males than females ($p<0.01$, data not shown). There were no main effects of trial or sex-by-trial interactions on Day 1 or Day 2 for the ratio of phospho-CREB^{Ser133} to total CREB (data not shown).

MTP

Protein expression for MTP was greater in females than males in sedentary control mice ($p<0.05$). On Day 1 and Day 2, there were no main effects of trial and no sex-by-trial interactions for MTP protein and mRNA expression (Figure 2-6).

Discussion

Accumulation of hepatic TG is associated with metabolic dysfunctions such as insulin resistance and elevated VLDL-TG secretion (33). Many of the benefits of habitual exercise are due to the most recent exercise bout rather than training-induced adaptations, however, little is known about the effects of acute exercise on hepatic TG metabolism and secretion and the influence of exercise intensity in males versus

females. In the present study, we examined the impact of single bouts of moderate-intensity CE versus HIIE on hepatic TG metabolism and secretion in female and male mice and our novel findings are as follows. Both CE and HIIE transiently increased hepatic TG concentration on Day 1 and in females the increase was greater with HIIE. Hepatic protein abundance of the lipid droplet coating protein PLIN2 was also transiently increased by exercise on Day 1 with a greater increase with HIIE in both sexes. Increases in PLIN2 protein with exercise were likely driven by increased gene transcription and the elevations in hepatic TG as well as PLIN2 protein and mRNA with exercise compared to CON on Day 1 were no longer present on Day 2 in both sexes. And finally, the reduced VLDL-TG secretion rate on Day 2 relative to CON which occurred only in females with HIIE was associated with increased hepatic TG content. Importantly, the differences between CE and HIIE occurred even despite bouts being identical in both total distance ran and bout duration and without differences between HIIE and CE for ad libitum food intake. These results reveal that both exercise intensity and sex alter hepatic TG metabolism and secretion following an acute bout of exercise. We also found greater basal VLDL-TG secretion rate in females, a sexual dimorphism present in humans that may be mediated by higher abundances of hepatic TG, AMPK, ACC, and MTP.

Our findings corroborate previous work with humans (32) and male mice (60) demonstrating increased hepatic TG after one bout of CE. In the present study, we extend these previous findings by demonstrating that the rise in hepatic TG with exercise can occur in both sexes and that the elevation largely subsides within ~28 hours

post-exercise, as shown by greater hepatic TG in CE and HIIE compared to CON within Day 1 and lack of differences between CE and HIIE vs. CON within Day 2. Of importance, we found sexual dimorphism in this response in that in females HIIE elicits an approximately 2-fold greater TG elevation than CE while in males both exercise modalities produce similar increases. To our knowledge, this is the first time it has been shown that sex differences in hepatic lipid metabolism in response to intense exercise exist. The transient elevation in hepatic TG appears not to be a result of increased de novo lipogenesis as no changes in ACC abundance or phosphorylation in response to exercise were observed. Enhanced adipose tissue lipolysis could result in increased delivery of FAs to the liver for esterification into TG. However, we did not detect any differences in plasma glycerol concentration, a marker of whole body lipolysis that closely tracks changes in plasma free fatty acid (FFA) concentration with exercise (51, 101, 123). It could be, however, that adipose lipolysis was elevated immediately after exercise but subsided prior to blood collection 3 hours later (20, 60).

Rather than simply a mass action effect from increased de novo lipogenesis or whole body lipolysis, it appears that the increases in hepatic TG with exercise could likely be related to PLIN2 expression and the sequestration of available FAs into hepatic lipid droplets, as an association between enhanced PLIN2 protein expression and hepatic TG was found. PLIN2 promotes hepatic TG storage likely by inhibition of intracellular TG lipolysis (14), while increases in hepatic TG could possibly elevate PLIN2 protein abundance through prevention of protein degradation (94). Because of this inter-dependence of PLIN2 protein and TG level, determining the direction of causality

between changes in PLIN2 protein and TG could be difficult in the absence of other measures. Thus, we measured expression of PLIN2 mRNA as well, and we discovered a strong significant positive correlation (R^2=0.95, p=0.001) between PLIN2 mRNA and protein expression levels. This finding suggests that in response to exercise, hepatic TG is increased through transcriptional regulation of PLIN2 expression. We also found that PLIN2 transcription with exercise is intensity-dependent, as evidenced by the statistically significant increase only with HIIE. The mechanism behind this is unknown, but it appears unrelated to hepatic AMPK signaling or PKA activation as measured by phosphorylation of AMPK and CREB, respectively. It could be related to exercise-induced PPARα activation or expression, as acute exercise stimulates its expression in the liver (65) and PPARα agonists stimulate hepatic PLIN2 expression (27), and this could be an important future area of investigation. In addition to the relationship between PLIN2 and hepatic TG with exercise, abundance of TG and PLIN2 protein also tended to track each other from Day 1 to Day 2 in CON, suggesting a possible but unconfirmed role of PLIN2 in changes in hepatic TG metabolism over time. Although our experiments were not designed to examine this time-related change in CON, future work may elucidate relationships between PLIN2 and hepatic TG under various conditions other than exercise.

We originally hypothesized that hepatic TG of exercised mice would be lower than CON. Thus, the transient increases in hepatic TG and PLIN2 did not support our original hypothesis. Reductions in hepatic TG with chronic exercise are thought to be related to improved insulin sensitivity, however, the role of hepatic TG in insulin

sensitivity is not entirely clear. Temporary rises in hepatic TG after acute exercise might also be beneficial, reflecting a buffering of FAs away from synthesis of potentially lipotoxic FA metabolites. Acute bouts of CE prevent lipid-induced whole-body insulin resistance by lowering concentrations of lipotoxic metabolites in skeletal muscle, partially by promoting storage of FAs as biologically inert TG (114). In addition, PLIN2 overexpression in muscle also increases insulin sensitivity in parallel with greater intramuscular TG (12). It could thus theoretically be possible that the transient increase in hepatic TG with exercise that appears to be caused by augmented PLIN2 expression promotes insulin sensitivity in the liver after exercise and this remains an interesting area for future research.

In agreement with a study in humans (9), exercise in the present study lowered VLDL-TG secretion in females. As well, the lack of changes in VLDL-TG secretion in males with CE and HIIE in our study is consistent with human studies showing that the effects of single bouts of prolonged or intense exercise on VLDL-TG kinetics in men are limited to only increased clearance (8, 92, 138, 139). Thus, our animal and exercise model used appears to be appropriate for inferring molecular underpinnings of human sex-differences in VLDL-TG kinetics with exercise. Compared to CON on Day 2, we found an approximately 20% decrease in VLDL-TG secretion rate with HIIE in females with no effect of CE and no impact of exercise on secretion on Day 1. Although HIIE resulted in attenuation of VLDL-TG secretion, we did not detect a change in plasma TG concentration, potentially because of the 2-fold greater inter-individual variability in plasma TG (38% vs. 20% between-animal coefficient of variation). As well, our kinetic

approach involved sampling blood TG at several time points for confirmation of complete blockade of circulating TG degradation and accurate calculation of secretion rate for each individual mouse (51) and therefore is a more robust measurement than determination of plasma TG concentration at a single time point.

Our original hypothesis was that reduced VLDL-TG secretion with HIIE would be mediated by AMPK activation, resulting in decreased hepatic TG. However, this potential mechanism was not supported by our findings. At first, our findings of unchanged AMPK phosphorylation by either exercise modality appear to be in disagreement with acute continuous exercise studies demonstrating increased hepatic AMPK activation with exercise and greater activation with high- vs. low-intensity exercise (20, 106). In these studies however, livers were collected immediately after exercise as opposed to the present study in which they were sampled 3 hours after. The mechanisms behind reduced VLDL-TG secretion in females with HIIE are unclear, but appear to be related to partitioning of FAs away from VLDL assembly and toward retention in lipid droplets. In the formation of VLDL particles, cytosolic TG undergoes lipolysis and FAs are transported into the endoplasmic reticulum for re-esterification into TG and incorporation into VLDL (86). Inhibition of this pathway theoretically would result in accumulation of TG in lipid droplets. Indeed, on Day 2 the reduction in VLDL-TG secretion in females was associated with a trend for a ~50% increase ($p=0.1$) in hepatic TG content. In addition, there was a significant inverse correlation between group changes in these two outcomes; correlations between group means rather than between individual data points should be interpreted with caution, but this finding may

further demonstrate a biological relationship between VLDL-TG secretion and hepatic TG concentration. Interestingly, although hepatic TG content was increased by exercise on Day 1 in both sexes, these changes were not associated with changes in VLDL-TG secretion at this time. Thus the relationship between hepatic TG content and secretion with exercise does not manifest relatively early in the post-exercise period but rather later during the following day. Glucagon (147) and other hormones such as catecholamines that would enhance PKA activity (16) are expected to stimulate lipolysis and thus promote transfer of FAs from lipid droplets to the endoplasmic reticulum in the liver. Additionally, MTP is required for incorporation of these FAs into VLDL. However, we found no changes in CREB phosphorylation (a marker of PKA activity (68, 69, 153)) or MTP abundance with CE or HIIE. While the reduction in VLDL-TG secretion with chronic exercise training has been attributable to decreased MTP (23), based on our findings the lowering of TG secretion with acute exercise is due to a different mechanism.

In addition to sex differences in hepatic TG metabolism and secretion in response to exercise, we found that our work on a common laboratory animal recapitulated human sex differences in the basal state. Females exhibited higher VLDL-TG secretion, which has been previously shown in humans (88, 98), as well as higher hepatic TG content. The reason for more TG in livers of female mice could be attributed to the two-fold greater ACC protein expression, as ACC catalyzes the formation of malonyl-CoA. Malonyl-CoA provides substrate for FA synthesis that can be incorporated into TG, and it also inhibits FA transport into the mitochondria. Thus, higher ACC

content can shift FAs away from oxidation and towards storage as TG. This can potentially lead to greater hepatic TG availability and provide higher FA supply via lipolysis for transport into the endoplasmic reticulum, re-esterification into TG, then through MTP incorporation into VLDL and secretion (86). As well, the higher hepatic AMPK content could play a role in this process by promoting TG lipolysis (50). Indeed, AMPK activation has been shown to stimulate hepatic TG secretion (108) and in the present study both AMPK and MTP were higher in sedentary females than males. Thus, it appears that females relative to males exhibit a hepatic enzyme profile that supports the synthesis and secretion of VLDL-TG.

In conclusion, we have discovered a novel metabolic impact of exercise in which transient alterations in hepatic TG metabolism are exhibited after exercise. The changes in hepatic lipid trafficking appear to be modulated by exercise-induced alterations in PLIN2 expression, and this response may be important for achieving health benefits of exercise or for adaptation to the stresses of exercise participation. Secondly, we have discovered potential mechanisms for sex differences in VLDL-TG secretion in the basal state and in response to a recent bout of intense exercise, and these results shed light upon sex-specific regulation of energy metabolism and the integration of metabolism between the liver and other tissues. As many of the effects of chronic exercise are expected to be a result of acute effects of each individual exercise bout, we expect that these results would provide information about certain aspects of the chronic exercise training response as well. In future work the effects of chronic training with these

exercise types (CE and HIIE) could be investigated to determine longer term effects on hepatic TG metabolism and ultimately the effect on metabolic health.

ACKNOWLEDGEMENTS

The authors thank Jesse Liou and Alex Burdzy for help with conducting exercise sessions, Dr. Judith Storch for use of the EchoMRI system, Dr. Judith Storch and Dr. John Douglass for technical advice regarding tyloxapol administration, Dr. Dawn Brasaemle for providing PLIN2 antibody and for critical commentary on results, and Dylan Klein for critical commentary on the manuscript.

GRANTS

This work was supported by the Faculty Research Grant Program of Rutgers University and by American Diabetes Association grant # 7-13-JF-27-BR.

DISCLOSURES

The authors declare no conflicts of interest.

Table 2-1. Characteristics of sedentary female and male mice.

	Females	Males
VLDL-TG secretion (mg/kg BW/min)	6.10±0.24	5.34±0.45[@]
VLDL-TG secretion (mg/dL/min)	5.54±0.22	4.86±0.41[@]
Plasma [TG] (mg/dL)	38±5	54±5[@]
Plasma [glycerol] (mM)	0.30±0.03	0.30±0.02
Body weight (g)	19.0±0.2	25.4±0.4*
Fat mass (g)	2.0±0.1	2.3±0.2
FFM (g)	17.1±0.2	23.1±0.3*
% Body fat	10.4±0.4	8.9±0.6[@]
Overnight food intake (g)	3.51±0.14	3.16±0.14

Values are means ± SE. Values shown for VLDL-TG secretion, plasma [TG], and plasma [glycerol] are from Day 1. *BW*, body weight. *FFM*, fat-free mass. Different from females: *$p<0.0001$, [@]$p<0.05$.

Figure 2-1

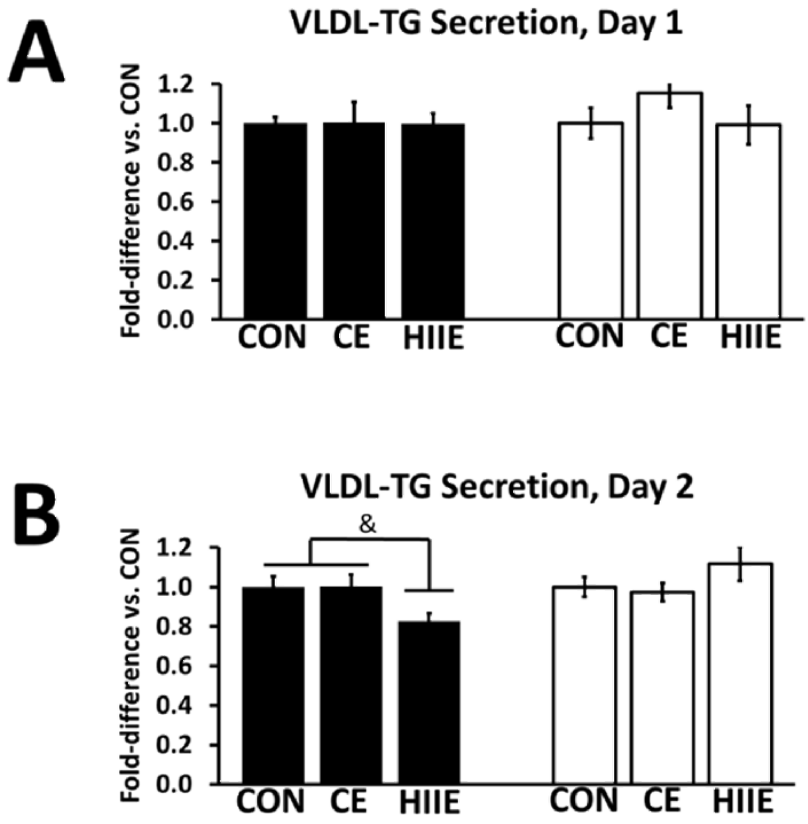

Figure 2-1. Relative differences in VLDL-TG secretion compared to CON of the same sex. Values are means ± SE. Females, black bars. Males, open bars. A: Day 1. No main effects of sex or trial and no sex-by-trial interaction. B: Day 2. Sex-by-trial interaction, $p<0.05$. $^{\&}$HIIE was significantly different from CE and CON in females, $p<0.05$.

Figure 2-2

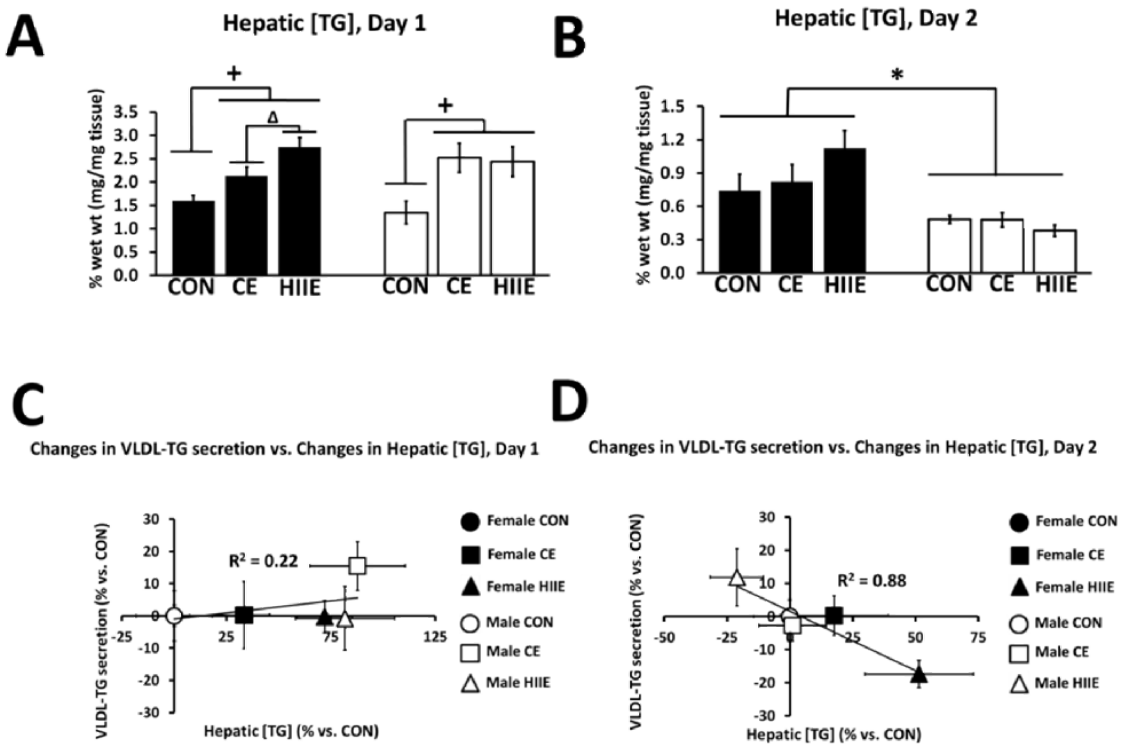

Figure 2-2. Hepatic TG concentrations. Values are means ± SE. Females, black bars. Males, open bars. A: Day 1. Main effect of trial, $p<0.0001$. $^{+}$CE and HIIE trials were significantly different from CON ($p<0.05$) but not from each other. No main effect of sex or sex-by-trial interaction. $^{\Delta}$The relative increase compared to CON was greater with HIIE than with CE ($p<0.05$). B: Day 2. *Main effect of sex, $p<0.0001$. No main effect of trial or sex-by-trial interaction. C: Group changes in VLDL-TG secretion and changes in hepatic [TG] compared to CON of same sex on Day 1. No significant correlation. D: Group changes in VLDL-TG secretion and changes in hepatic [TG] compared to CON of same sex on Day 2. Significant inverse correlation ($R^2=0.88$, $p=0.0058$).

Figure 2-3

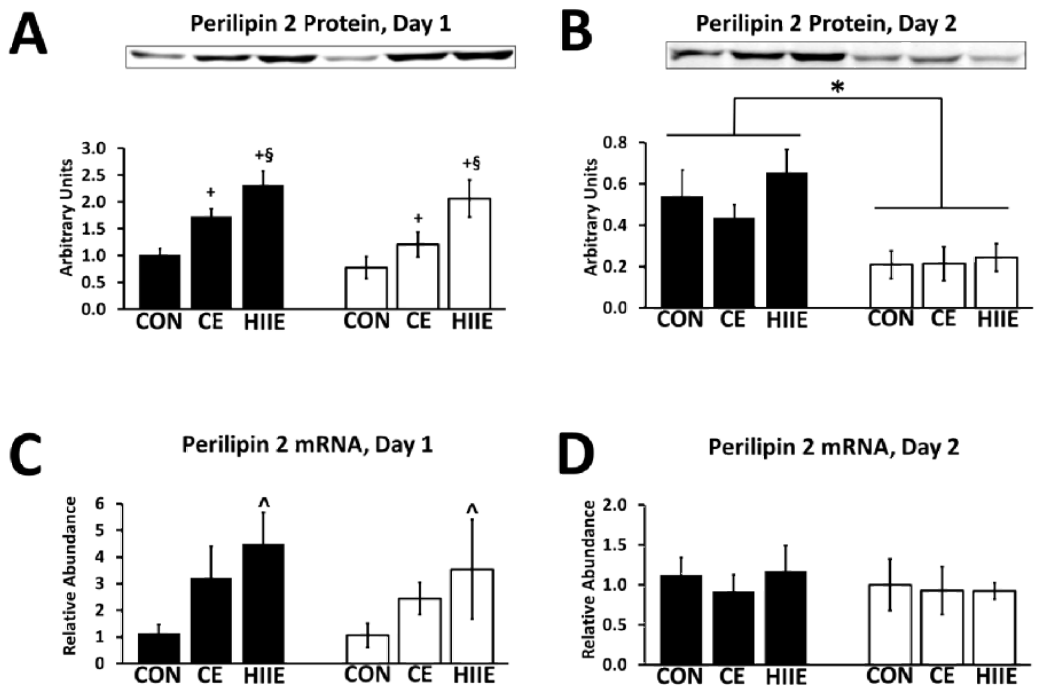

Figure 2-3. Hepatic PLIN2 expression. Values are means ± SE. Females, black bars. Males, open bars. A: Protein abundances on Day 1. Main effect of trial, $p<0.0001$. $^{+}$Significantly different from CON, $p<0.05$. §Significantly different from CE, $p<0.05$. No main effect of sex or sex-by-trial interaction. B: Protein abundances on Day 2. *Main effect of sex, $p=0.0001$. No main effect of trial or sex-by-trial interaction. C: mRNA abundances on Day 1. Main effect of trial, $p<0.05$. $^{\wedge}$Significantly different from CON, $p<0.05$. No main effect of sex or sex-by-trial interaction. D: mRNA abundances on Day 2. No main effects of sex or trial and no sex-by-trial interaction.

Figure 2-4

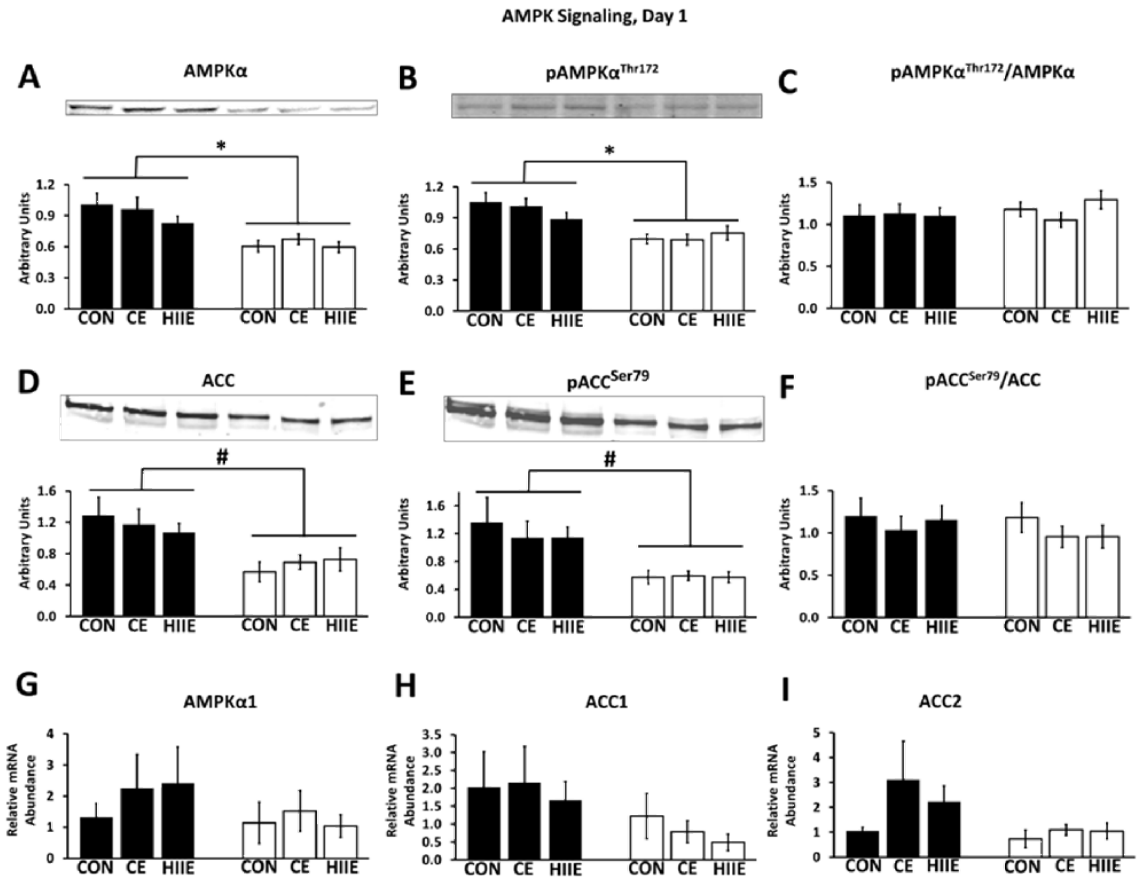

Figure 2-4. Hepatic AMPK signaling and related protein and gene expression on Day 1. Values are means ± SE. Females, black bars. Males, open bars. A: Total AMPKα. *Main effect of sex, p<0.0001. No main effect of trial or sex-by-trial interaction. B: Phosphorylated AMPKα^{Thr172}. *Main effect of sex, p<0.0001. No main effect of trial or sex-by-trial interaction. C: Ratio of phosphorylated AMPKα^{Thr172} to total AMPKα. No main effects of sex or trial and no sex-by-trial interaction. D: Total ACC. #Main effect of sex, p<0.001. No main effect of trial or sex-by-trial interaction. E: Phosphorylated ACC^{Ser79}. #Main effect of sex, p<0.001. No main effect of trial or sex-by-trial interaction. F: Ratio of phosphorylated ACC^{Ser79} to total ACC. No main effects of sex or trial and no sex-by-trial interaction. G-I: mRNA expression of AMPKα1, ACC1, and ACC2, respectively. No main effects of sex or trial and no sex-by-trial interaction.

Figure 2-5

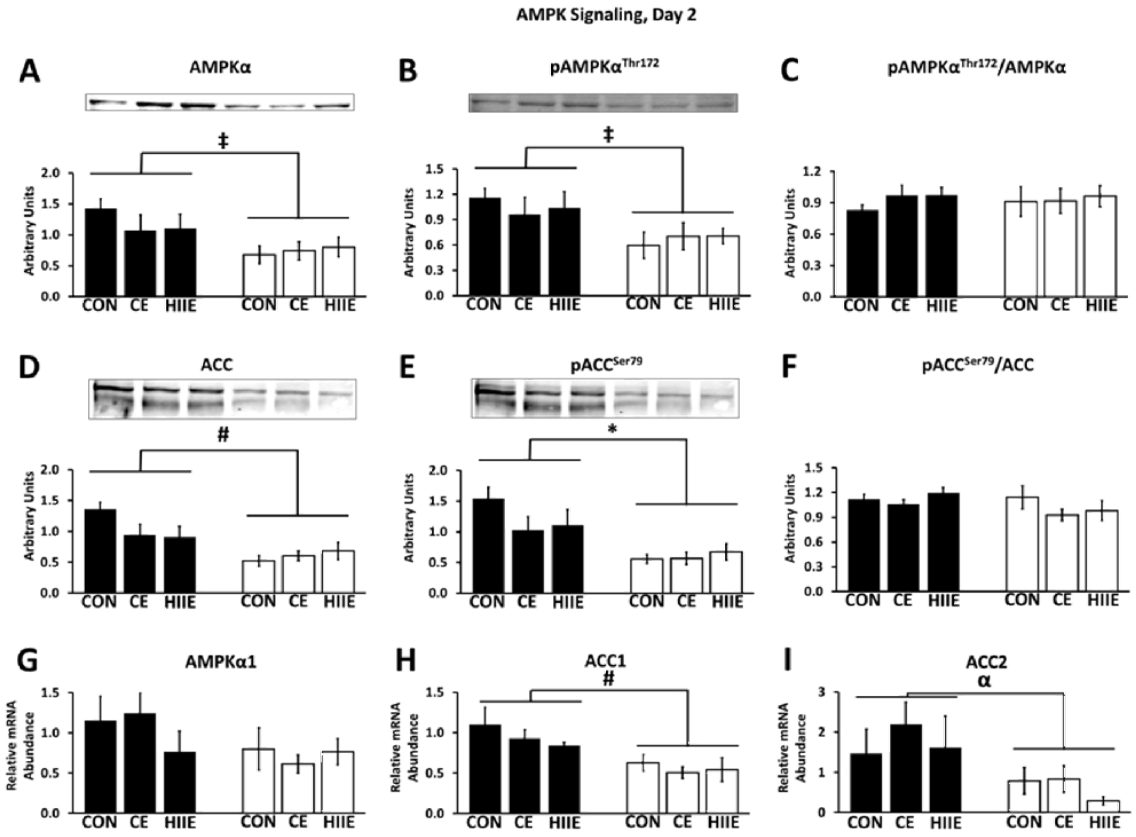

Figure 2-5. Hepatic AMPK signaling and related protein and gene expression on Day 2. Values are means ± SE. Females, black bars. Males, open bars. A: Total AMPKα. ‡Main effect of sex, p<0.01. No main effect of trial or sex-by-trial interaction. B: Phosphorylated AMPKα^{Thr172}. ‡Main effect of sex, p<0.01. No main effect of trial or sex-by-trial interaction. C: Ratio of phosphorylated AMPKα^{Thr172} to total AMPKα. No main effects of sex or trial and no sex-by-trial interaction. D: Total ACC. #Main effect of sex, p<0.001. No main effect of trial or sex-by-trial interaction. E: Phosphorylated ACC^{Ser79}. *Main effect of sex, p=0.0001. No main effect of trial or sex-by-trial interaction. F: Ratio of phosphorylated ACC^{Ser79} to total ACC. No main effects of sex or trial and no sex-by-trial interaction. G: mRNA expression of AMPKα1. No main effects of sex or trial and no

sex-by-trial interaction. H-I: mRNA expression of ACC1 and ACC2, respectively. Main effects of sex, $^{\#}p<0.001$, $^{\alpha}p<0.05$. No main effects of trial or sex-by-trial interactions.

Figure 2-6

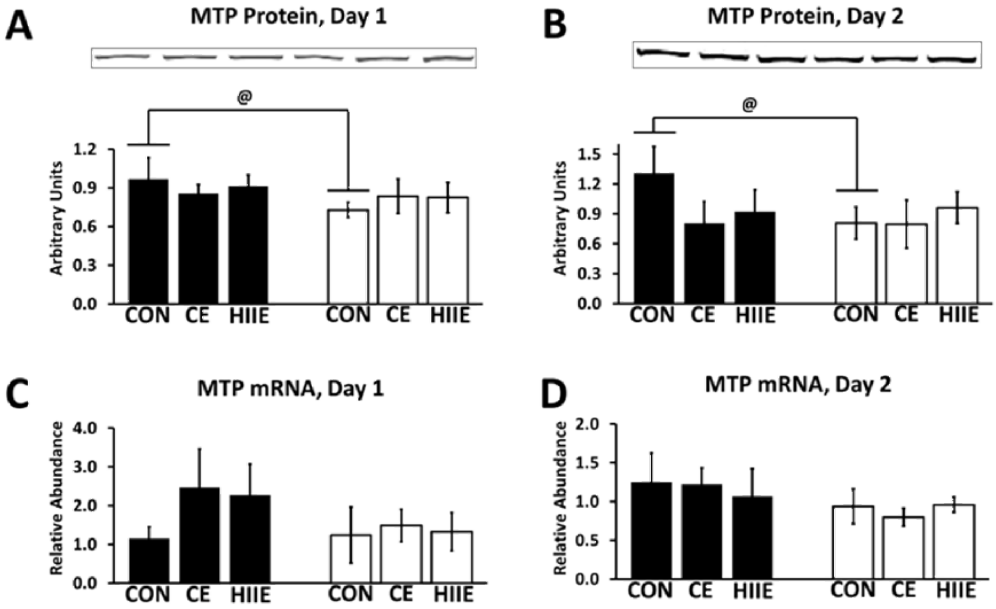

Figure 2-6. Hepatic MTP expression. Values are means ± SE. Females, black bars. Males, open bars. A: Protein abundances on Day 1. [@]Significantly greater in females than males in sedentary control mice ($p<0.05$). No main effect of trial or sex-by-trial interaction. B: Protein abundances on Day 2. [@]Significantly greater in females than males in sedentary control mice ($p<0.05$). C: mRNA abundances on Day 1. No main effects of sex or trial and no sex-by-trial interaction. D: mRNA abundances on Day 2. No main effects of sex or trial and no sex-by-trial interaction.

Chapter 3: Effects of ovariectomy and exercise training intensity on whole body energy substrate metabolism, hepatic lipid metabolism, and behavior in mice

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Author contributions:

M.A.T. and G.C.H. conception and design of research; M.A.T. performed experiments with assistance from S.C.C. and D.J.K.; M.A.T. analyzed data; M.A.T., S.C.C., T.G.A.. M.U., and G.C.H. interpreted results of experiments; M.A.T. prepared figures; M.A.T. drafted the manuscript.

Abstract

Menopause is associated with adverse effects including fatty liver, blood glucose dysregulation, increased fat mass, and decreased muscular strength. Previously, we demonstrated that single sessions of high-intensity interval exercise (HIIE) are more effective than distance- and duration-matched continuous exercise (CE) at altering hepatic triglyceride (TG) metabolism and very-low density lipoprotein-TG (VLDL-TG) secretion. In the present study, we compared 6 weeks of training using these exercise modalities on hepatic TG metabolism and secretion, glucose tolerance, body composition, and grip strength in ovariectomized (OVX) and sham-operated (SHAM) mice. OVX and SHAM were assigned to CE, HIIE, or sedentary control. HIIE was a 30-45min session of 30s running intervals (30m/min) interspersed with 60s rest periods (5m/min). CE was a distance- and duration-matched run at 13.8m/min. Energy expenditure during exercise bouts was the same between CE and HIIE and both types of exercise similarly reduced absolute carbohydrate oxidation rate and spontaneous physical activity (SPA) in the post-exercise period. OVX vs. SHAM displayed impaired glucose tolerance, elevated fasting blood glucose, and greater body fat despite lower hepatic TG, as well as lower strength, and these outcomes were not affected by training. Neither type of training altered VLDL-TG secretion. Only HIIE increased protein abundance of hepatic AMP-activated protein kinase (AMPK) in OVX and SHAM. Our results reveal intensity-dependent effects on hepatic AMPK expression as well as effects of exercise on subsequent SPA and substrate oxidation. These findings may have

implications for designing training prescriptions for postmenopausal women and other populations.

Introduction

Menopause is associated with numerous adverse health effects including insulin resistance (74, 85), increased fat mass (40, 58, 70, 81, 85, 129, 134), and loss of muscular strength (67), and exercise is an attractive countermeasure due to its potential to favorably alter lipid and glucose metabolism. High-intensity interval exercise (HIIE), which involves alternating between relatively easy and challenging intensities within a single exercise bout, has been reported to be more enjoyable than traditional moderate-intensity continuous exercise (CE, e.g., jogging at constant speed) (6) and is gaining much popularity. Because of the potential metabolic effects of the bursts of highly intense activity, training with this type of exercise may be a more effective strategy than CE for treating metabolic dysregulation that can accompany menopause. It is possible that HIIE could lower hepatic TG to a greater extent than CE through activation of hepatic AMP-activated protein kinase (AMPK). Enhanced AMPK activity could result in lower acetyl-CoA carboxylase (ACC) and higher malonyl-CoA decarboxylase activities, resulting in decreased malonyl-CoA content (106). Malonyl-CoA is a substrate for fatty acid (FA) synthesis and an inhibitor of FA transport into the mitochondria, and so a reduction in its concentration could promote partitioning of FAs toward oxidation and away from esterification into TG. Hepatic AMPK and ACC are

activated and deactivated, respectively, to greater extents by single bouts of high- compared to low-intensity exercise (20); thus, it is plausible that chronic exposure to HIIE may have larger impacts on hepatic TG metabolism than CE. Further, chronic HIIE has been shown to be more effective than CE in promoting fat loss despite similar (135) or lower (136) energy expenditures. However, the possibility of HIIE being a better strategy than CE for favorably altering body composition and lipid metabolism in ovarian hormone deficiency remains to be explored.

Chronic HIIE has been shown to reduce hepatic very-low density lipoprotein-TG (VLDL-TG) secretion in men (137). Additionally, we have shown recently that a single bout of HIIE but not CE lowers VLDL-TG secretion rate in female mice (142). VLDL-TG transports lipid to peripheral tissues including adipose and skeletal muscle, thus repeated reductions in secretion that could potentially occur with habitual exercise could possibly lower uptake of FAs derived from VLDL-TG resulting in lower adipose mass as well as improved insulin sensitivity by reducing lipid accumulation in muscle (104). Indeed, mouse models show that the majority of FA flux into adipose tissue is derived from plasma TG rather than FFA (144), and alterations in hepatic lipid metabolism can influence adiposity (99) and whole-body glucose tolerance (36).

In addition to effects on VLDL-TG secretion (137, 142), HIIE may also have beneficial effects on muscle strength. In a study with elderly men, chronic HIIE (4 min at 65-75% VO_{2peak} and 5^{th} minute at 85-95% VO_{2peak} repeated 7 times for a total of 45 min) increased Type IIA muscle fiber cross-sectional area (24). Furthermore, CE training at

80% of maximal heart rate in elderly women and men also increased Type IIA as well as Type I muscle fiber cross-sectional area (26). As muscle force production capability is directly proportional to cross-sectional area, it could be expected that these types of adaptations to high intensity exercise may lead to some increase in muscular strength. However, whether HIIE actually increases functional strength and is more effective than CE remains unknown.

The ovariectomized mouse is an animal model of menopause that recapitulates many of the impairments that post-menopausal women experience (42, 102, 112, 125, 155) and can be used for preclinical study of novel intervention strategies. Because of the potential for exercise training to attenuate severity of complications associated with menopause and the possible higher effectiveness of HIIE vs. CE, we compared 6 weeks of training using these exercise types on hepatic TG metabolism and secretion, glucose tolerance, body composition, and grip strength in ovariectomized and sham-operated mice. In addition, we measured energy expenditure and substrate oxidation both during and immediately after exercise as well as assessed post-exercise spontaneous physical activity (SPA) to further characterize physiological and behavioral responses to these exercise modalities.

Methods

Animals. This protocol was approved by the Institutional Animal Care and Use Committee of Rutgers University. Female C57BL/6J mice (Jackson Laboratory, Bar

Harbor, ME) were maintained on a 12-hour light/dark photoperiod with all mice allowed ad libitum access to food and water. Mice consumed the Labdiet 5K52 diet (Purina Mills, Richmond, IN) for their lifetimes and were acclimated to the facility for at least 1 wk prior to surgery.

Surgical procedures. Surgeries were performed between 14 and 15 weeks of age 10 days before the start of exercise training. On the day of surgery, mice were assigned to ovariectomy (OVX) or sham-operation (SHAM), matched for bodyweight. Mice were maintained under anesthesia with isoflurane and incision sites were shaved and cleaned with isopropanol and betadine followed by injection with marcaine. Bilateral incisions were made, and the uterine horn just below the ovary was sutured followed by ovary extraction. For SHAM mice, ovaries were raised through the incision site and lowered back into the animal. Incisions were closed using wound clips. Immediately after surgery, mice were administered ibuprofen (7.5 mg/kg) orally.

Exercise protocols and training progression. Mice ran on a treadmill (Exer-3/6, Columbus Instruments, Columbus, OH) with a shock grid set at minimal intensity (1 on a scale of 0-10). Immediately before the start of training, mice within SHAM and OVX were assigned to CE, HIIE, or time-matched sedentary controls (CON), matched for body weight. Initially, CE and HIIE bouts were 30 minutes in duration following a 5 min warmup at 5 m/min with both the warmup and exercise performed at an incline of 25° as done previously by our group (142). For HIIE, mice ran for 30 s sprint intervals interspersed with 60 s walking rest periods at 5 m/min. The first, second, and third

sprint intervals were at 15, 20, and 25 m/min, respectively, followed by all remaining sprint intervals at 30 m/min. Acceleration to final sprint interval speeds was performed within 5 s and deceleration back to 5 m/min within 2 s. CE bouts were distance-, duration-, and incline- matched runs at a constant speed of 13.8 m/min. Mice were prodded gently when closely approaching the shock grid to minimize shocks and to maintain speed. Duration and frequency of exercise were increased progressively as follows. Weeks 1-2: 3 days, 30 min/session; weeks 3-4: 4 days, 30 min/session; weeks 5-6: 4 days, 45 min/session. CON mice performed a 5 min walk at 5 m/min on the same days as CE and HIIE bouts to control for potential stress of repeated handling and placement into the treadmill. Training (45 min/session) continued into a 7^{th} week of exercise participation as needed such that VLDL-TG secretion rates could be measured and tissues collected on the day after the most recent exercise bout when it coincided with the diestrus stage of the estrous cycle in SHAM mice (to control for potential effects of ovarian hormone variation in SHAM). When a SHAM mouse was assessed for VLDL-TG secretion or killed for tissue collection, a corresponding OVX mouse (CON, CE, or HIIE) was assessed at that time as well. The final exercise bout of training was conducted between 11:00am and 1:00pm, approximately the same time of day as our previous study examining the acute effects of these exercise bouts (142).

Body composition and food intake. Body weights and food intake were measured weekly starting 1 week before the onset of training and body composition analyzed by EchoMRI (Echo Medical Systems, Houston, TX) at the start of training and every 2 weeks thereafter.

Indirect calorimetry and spontaneous physical activity. An Oxymax/CLAMS unit (Columbus Instruments) was used for indirect calorimetry and post-exercise SPA assessment. Indirect calorimetry during exercise (25° incline, 5 min at 5 m/min plus 30 min exercise as described above) was conducted after a 2 hr fast on an enclosed treadmill (Columbus Instruments) after a day of no exercise during the 3rd or 4th week of training and gas exchange was measured continuously. For CON, the treadmill remained stationary and indirect calorimetry was conducted for the same duration as CE and HIIE. In order to capture the acute responses that would occur after exercise following the habitual training sessions, post-exercise indirect calorimetry and SPA measurements were performed for 4 hours immediately after exercise during the 2nd or 3rd week of training after a day of no exercise. After a 2 hr fast, mice were exercised (25° incline, 5 min at 5 m/min plus 30 min exercise as described above) immediately followed by placement into indirect calorimetry chambers with free access to water and no food. Gas exchange was measured every 10 min and spontaneous activity was counted as the total sum of x-axis IR beam breaks (x total activity), successive x-axis IR beam breaks (x ambulatory activity), and vertical IR beam breaks (z total activity). Respiratory exchange ratio (RER) was calculated as the ratio of carbon dioxide production (VCO_2) to oxygen consumption (VO_2) and averaged over the course of measurement. Metabolic rate was calculated as [3.815 + (1.232 x RER)] x VO_2 (mL/kg BW/hr) (84), averaged over the course of measurement, and expressed as an absolute rate (kcal/hr) and normalized to bodyweight (kcal/kg BW/hr) and fat-free mass (FFM)

(kcal/kg FFM/hr). Oxidation of carbohydrate and lipid (83) over the course of measurement was averaged and expressed as absolute rates (kcal/hr).

Estrous stage determination. Estrous stage in SHAM mice was tracked by daily evaluation of vaginal smears (61) during the entire 6^{th} week of training up to and including the day mice were killed in the 7^{th} week during the diestrus stage. To control for the potential stress of the procedure, vaginal smears were simultaneously taken from SHAM and OVX.

Hepatic TG secretion. VLDL-TG secretion rate measurement was conducted the day after the most recent exercise bout at the end of the chronic exercise training period. As previously done by our group (142), food was withdrawn at 7:00AM on the day of measurement. Following an 8 hr fast, blood TG rate of appearance (considered to represent hepatic VLDL-TG secretion rate), was measured as detailed previously (51, 142). Using a hand-held meter (Cardiochek, Polymer Technology Systems, Inc., Indianapolis, IN), measurement of blood TG was performed on samples (approximately 15 µL) taken at 20-min intervals from the tail after intraperitoneal injection of tyloxapol (500 mg/kg) (Sigma-Aldrich, St. Louis, MO) until concentration exceeded 500 mg/dL. Tyloxapol inhibits breakdown of circulating TG such that blood [TG] increases linearly and the slope of the linear regression represents VLDL-TG secretion rate (51). Linearity ($R^2 \approx 0.99$) was achieved consistently. Rates were expressed as per unit blood volume (mg/dL/min) and normalized to bodyweight (mg/kg BW/min) based on blood volume (116). After measurement, mice were euthanized by CO_2 inhalation.

Tissue collection. Tissues were collected the day after the most recent exercise bout, after the completion of the chronic exercise training period, in a separate set of mice than those used for VLDL-TG secretion measurement. Again, food was withdrawn at 7:00AM on the day of collection and tissues taken at the same time of day as VLDL-TG secretion measurement. Mice were euthanized by CO_2 inhalation and immediately after, blood was collected by cardiac puncture in EDTA-coated tubes followed by isolation of plasma and storage at -80°C until analysis. Liver samples were quickly collected right after blood draw, immediately frozen in liquid nitrogen, then stored at -80°C until analysis. After liver was collected and frozen, gonadal and inguinal fat pads, quadriceps muscle, and uteri were extracted and immediately frozen in liquid nitrogen.

Biochemical assays. Plasma glycerol (Sigma-Aldrich), TG (Sigma-Aldrich), and insulin (EMD Millipore Corporation, St. Charles, MO) concentrations were measured by commercially available kits. Concentration of hepatic TG was determined by isolation of lipids from approximately 8 mg of tissue and measurement of TG using a commercially available kit (Sigma-Aldrich) (37, 54, 142) and expressed as percentage of liver wet weight (mg/mg tissue).

Oral glucose tolerance test (OGTT) and fasting blood glucose. Blood glucose concentrations were measured using a handheld meter (Precision Xtra, Abbott Laboratories, Abbot Park, IL). OGTTs were performed during the 6^{th} week of training on a day after exercise at least 3 days prior to VLDL-TG secretion measurement and tissue collection. Exercise on the day before OGTTs was conducted between 11:00AM and

1:00PM and no exercise was performed on the day of testing. OGTTs were performed at 3:00PM after a 5 hour fast. After administration of glucose orally (2 g/kg BW with a 20% solution in water), glucose was measured on blood samples from the tail immediately after ingestion and 10, 20, 30, 60, 90, and 120 minutes after, and area under the curve (AUC) was calculated using the trapezoidal rule. For fasting blood glucose, samples from the tail were measured at 2:00PM prior to VLDL-TG secretion measurement and tissue collection.

Grip strength. Grip strength was assessed as previously (54, 76, 141) on the first day of the 7th week of training prior to any exercise. Mice were held by the tail and lowered onto an angled-mesh grid attached to a force transducer (Columbus Instruments), pulled toward the force transducer until their grips released, and peak tension (grams force) was recorded. Mice performed 5 attempts in immediate succession and the 3 highest attempts were averaged. Results were expressed as absolute force (g force) as well as normalized to bodyweight (g force/g BW) and FFM (g force/g FFM).

Western blotting. Western blotting was conducted as previously (54, 142) with slight modifications. Liver samples (~40 mg) were homogenized followed by gel electrophoresis of 50 μg protein and transfer onto membranes. Membranes were incubated with the following primary antibodies: mouse anti-mouse total AMPKα (1:400; Cell Signaling Technology, Danvers, MA), rabbit anti-mouse phospho-AMPKα^{Thr172} (1:800; Cell Signaling Technology), rabbit anti-mouse total ACC (1:8,000; Abcam, Cambridge, MA), rabbit anti-mouse phospho-ACC^{Ser79} (1:1,000; Cell Signaling

Technology), rabbit anti-mouse PLIN2 (1:25,000; kindly provided by Dr. Dawn Brasaemle of Rutgers University), mouse anti-mouse microsomal triglyceride transfer protein (MTP;1:100,000; BD Transduction Laboratories, San Jose, CA), rabbit anti-mouse phospho-p65 as an inflammatory marker (143) (1:1000; Cell Signaling Technology), and rabbit anti-mouse β-actin (1:5,000; Cell Signaling Technology) as a loading control. After primary antibody incubations, membranes were then incubated with goat anti-rabbit IR Dye 680 (1:10,000; LI-COR Biosciences, Lincoln, NE), goat anti-mouse IR Dye 800 (1:10,000; LI-COR Biosciences), and goat anti-rabbit IR Dye 800 (1:10,000; LI-COR Biosciences) secondary antibodies and bands quantified (54, 142).

mRNA quantitation. mRNA isolation and quantitation was performed as previously (18, 142) with minor adjustment. Briefly, liver samples (~35 mg) were pulverized under liquid nitrogen temperature. RNA was extracted (18) then treated with RNAsecure (Life Technologies, Grand Island, NY) and DNase 1 (Life Technologies). Synthesis of cDNA was carried out using a commercially available kit (Life Technologies) and RT-PCR with the following TaqMan Gene Expression Assays (Life Technologies): PLIN2 (Assay ID Mm00475794_m1), AMPKα1 (Assay ID Mm01296700_m1), ACC1 (Assay ID Mm01304257_m1), ACC2 (Assay ID Mm0120467_m1), MTP (Assay ID Mm00435015_m1), fibroblast growth factor 21(FGF21; Assay ID Mm00840165_g1) and 18s ribosomal RNA (cat. no. 4352930E) as the endogenous control as detailed previously (8). Results are expressed as fold-difference relative to CON mice in SHAM.

Statistical analysis. Data are presented as means ± SE. Effects of ovarian hormone deficiency (surgery) and type of training were analyzed by 2-way (surgery-by-training) ANOVA. Comparisons across time points were made by repeated measures 3-way (surgery-by-training-by-time) ANOVA for bodyweight, fold-change in percent fat mass, food intake, and OGTTs. For fat mass and body fat percentage, repeated measures 3-way (surgery-by-training-by-time) ANCOVA with baseline measurements as covariates was used. ANOVA and ANCOVA were followed by Fisher's Least Significant Difference (LSD) post hoc test. JMP version 10 (SAS Institute Inc., Cary, NC) was used for statistical analyses and alpha less than 0.05 was considered statistically significant.

Results

Uterine weights. In OVX vs. SHAM uterine weights were dramatically reduced (20 vs. 80 mg; main effect of surgery, $p<0.0001$), indicating successful removal of ovaries and induction of ovarian hormone deficiency.

Indirect calorimetry during exercise. VO_2 and metabolic rate (Figures 3-1A and 3-1B) were both significantly lower in OVX compared to SHAM when normalized to bodyweight (main effect of surgery, $p<0.05$ for VO_2 and metabolic rate) and when normalized to FFM (main effect of surgery, $p<0.01$ for VO_2 and $p<0.05$ for metabolic rate; data not shown). When expressed as absolute rates (mL/hr and kcal/hr for VO_2 and metabolic rate, respectively), there were no significant differences between OVX and SHAM (data not shown). Main effects of training ($p<0.0001$) were observed for VO_2

and metabolic rate expressed as absolute rates as well as normalized to bodyweight and FFM with post hoc testing indicating that CE and HIIE were both greater than CON but not different from each other ($p<0.05$). For RER (Figure 3-1C), there was a significant main effect of training ($p<0.0001$) with post hoc analysis showing that both CE and HIIE were higher than CON ($p<0.05$) during exercise with a significantly greater elevation with HIIE than CE ($p<0.05$). Absolute carbohydrate oxidation was also higher in CE and HIIE than CON ($p<0.05$) but not different from each other (main effect of training, $p<0.0001$; Figure 3-1D). In OVX, absolute carbohydrate oxidation tended to be higher in HIIE vs. CE ($p=0.045$ by t-test). For absolute lipid oxidation, there were no main effects of surgery or training (Figure 3-1E). No surgery-by-training interactions were observed for VO_2, metabolic rate, RER, or for absolute carbohydrate and lipid oxidation.

Post-exercise indirect calorimetry and SPA. VO_2 ($p<0.05$) and metabolic rate ($p=0.05$) were both lower in OVX compared to SHAM when normalized to bodyweight (main effect of surgery, $p<0.05$; Figures 3-2A and 3-2B) but not when normalized to FFM (main effect of surgery, $p=0.20$; data not shown). When expressed as absolute rates, VO2 ($p<0.05$) and metabolic rate ($p<0.05$) were slightly higher (~5-6%) in OVX compared to SHAM (main effect of surgery, $p<0.05$; data not shown). There were no main effects of training for VO_2 or metabolic rate expressed as absolute rates or normalized to bodyweight or FFM. RER in CE and HIIE were both significantly lower than CON during post-exercise recovery ($p<0.05$) but not different from each other (main effect of training, $p<0.0001$; Figure 3-2C). However, RER tended to be lower in HIIE compared to CE ($p=0.10$) in SHAM. A main effect of training ($p<0.0001$) was observed for absolute

carbohydrate oxidation with post hoc testing indicating that both CE and HIIE were lower than CON ($p<0.05$) but not different from each other (Figure 3-2D). The rate of absolute lipid oxidation was approximately 6% greater in OVX vs. SHAM (main effect of surgery, $p<0.05$) with no main effect of training. There were significant main effects of training on X total ($p<0.01$), X ambulatory ($p<0.01$), and Z total ($p<0.05$) activities with post hoc testing showing that both CE and HIIE were lower than CON ($p<0.05$) but not different from each other for each type of activity (Figures 3-2D-F). There were no surgery-by-training interactions for VO_2, metabolic rate, RER, absolute carbohydrate and lipid oxidation, or SPA.

Body composition and food intake. Bodyweights (Figure 3-3A) were significantly greater in OVX compared to SHAM overall over the course of training (main effect of surgery, $p<0.0001$) with significant differences between OVX and SHAM (surgery-by-time interaction, $p<0.0001$) at each week from week 0-6 ($p<0.05$). Absolute fat mass (Figure 3-3B) was elevated in OVX overall (main effect of surgery, $p<0.01$) and specifically at weeks 2-6 (surgery-by-time interaction, $p<0.01$) vs. SHAM ($p<0.05$). Qualitatively similar results were found for body fat percentage (data not shown) with a trend for OVX to be higher overall (main effect of surgery, $p=0.07$) and significantly greater ($p<0.05$) at weeks 4 and 6 (surgery-by-time interaction, $p<0.01$). Similarly, relative increases in fat mass over time (fold-increase from baseline) (Figure 3-3C) were greater in OVX vs. SHAM overall (main effect of surgery, $p<0.01$) as well as specifically at weeks 4 and 6 ($p<0.05$) (surgery-by-time interaction, $p<0.001$). No main effects of surgery or training and no surgery-by-training interaction were found for gonadal and inguinal fat pad and

quadriceps weights (data not shown). Weekly food intake was lower in OVX vs. SHAM when normalized to bodyweight (main effect of surgery, $p<0.0001$; Figure 3-3D) and FFM (main effect of surgery, $p<0.0001$; data not shown) but no differences were found when expressed as absolute intake (data not shown). Results from analysis of food intake indicated that there were no main effects of training and no interactions between factors.

Oral glucose tolerance, fasting blood glucose, and plasma insulin. Blood glucose concentration was higher in OVX vs. SHAM overall (main effect of surgery, $p<0.01$) and specifically at 10, 20, 30, and 60 min ($p<0.05$) after glucose administration (surgery-by-time interaction, $p<0.01$) with no main effect of training or surgery-by-training interaction (Figure 3-3E). AUC (Figure 3-3F; main effect of surgery, $p<0.01$) and fasting blood glucose (Table 3-1; main effect of surgery, $p<0.05$) were also greater in OVX vs. SHAM. Plasma insulin concentration in SHAM was similarly reduced by both types of training ($p<0.05$) compared to CON, and in OVX was lower in CE than HIIE ($p<0.05$) (surgery-by-training interaction, $p<0.05$).

TG kinetics. There were no significant main effects of surgery or training and no interactions for VLDL-TG secretion, plasma TG concentration, and plasma glycerol concentration (Table 3-1). Plasma glycerol tended to be lower in OVX vs. SHAM (main effect of surgery, $p=0.09$). In SHAM, there was a trend for plasma glycerol in HIIE to be lower compared to CON ($p=0.09$), reducing it approximately to the level seen in OVX mice.

Hepatic TG and PLIN2. Hepatic TG ($p<0.01$; Table 3-1) and PLIN2 protein ($p<0.05$; Figure 3-4A) content were significantly lower in OVX compared to SHAM (main effects of surgery). There were no main effects of training and no surgery-by-training interactions for either hepatic TG or PLIN2 protein. For PLIN2 mRNA, there were no main effects of surgery or training and no surgery-by-training interaction.

Hepatic AMPK signaling and related gene and protein expression. Abundance of total AMPKα protein was increased only by HIIE ($p<0.05$) compared to CON (main effect of training, $p<0.05$) with no main effect of surgery or surgery-by-training interaction (Figure 3-5A). AMPKα mRNA data followed a similar pattern, but exercise effects did not reach statistical significance. The ratio of phosphorylated AMPKα^{Thr172} to total AMPKα was significantly lower ($p<0.05$) in HIIE vs. CON (main effect of training, $p<0.05$) and significantly lower in OVX compared to SHAM (main effect of surgery, $p<0.05$) with no surgery-by-training interaction (Figure 3-5C). There were no main effects of surgery or training and no surgery-by-training interactions for pAMPKα^{Thr172}, ACC, pACC^{Ser79}, and the ratio of pACC^{Ser79} to total ACC as well as for mRNA abundances of ACC1 and ACC2.

MTP, FGF21, and phosphorylated p65. MTP protein abundance tended to be lower in OVX vs. SHAM (main effect of surgery, $p=0.11$; Figure 3-4C). No main effect of training or surgery-by-training interaction was observed for MTP protein. There were no significant main effects of surgery or training and no surgery-by-training interactions for MTP (Figure 3-4D), FGF21 mRNA, or for phosphorylated p65 (data not shown). FGF21,

however, tended to be approximately 50% lower in OVX compared to SHAM (main effect of surgery, $p=0.15$)

Grip strength. Grip strength (Figure 3-6) expressed as absolute force (main effect of surgery, $p<0.05$) as well as force normalized to bodyweight (main effect of surgery, $p<0.0001$) and FFM (main effect of surgery, $p=0.0001$; data not shown) were lower in OVX vs. SHAM. No main effects of training or surgery-by-training interactions were observed.

Discussion

Menopause is associated with numerous adverse outcomes including insulin resistance (74, 85), increased fat mass (40, 58, 70, 81, 85, 129, 134), and loss of muscular strength (67). Exercise training is an attractive approach for helping to prevent these negative health effects, however, the relative efficacies of different exercise modalities is largely unknown. We characterized CE and HIIE in terms of whole-body energy expenditure and substrate oxidation and examined the effects of 6 weeks of training with these exercise modalities on hepatic TG metabolism, glucose tolerance, body composition, and strength in ovariectomized and sham-operated mice, and our major findings are as follows. In OVX and SHAM, CE and HIIE elicited similar energy expenditures during exercise bouts and in the post-exercise period lowered absolute carbohydrate oxidation in association with reduced SPA. OVX displayed impaired glucose tolerance, elevated fasting blood glucose, and greater body fat despite lower

hepatic TG, as well as lower strength, relative to SHAM and these outcomes were not affected by training. In contrast with acute exercise responses to these exercise modalities (142), chronic HIIE led to increased hepatic AMPK protein expression. Another difference between a single session of HIIE (142) vs chronic HIIE (present results) is that the acute reduction in VLDL-TG secretion after exercise is not maintained after 6 weeks of training. Our results reveal intensity-dependent effects of chronic exercise training on hepatic AMPK expression and together with previous findings reveal distinct responses in hepatic TG metabolism and secretion to acute vs. chronic exercise.

Ovariectomy in mice has been shown to elevate (42, 112, 155) or have no effect (63) on hepatic TG content. In the present work, hepatic TG in OVX was moderately reduced relative to SHAM and this was consistent with our finding of decreased protein abundance of the lipid-droplet coating protein PLIN2, which is known to track TG levels in liver (142) and muscle (119, 120). In another study, ovariectomized mice fed a high-fat but not normal diet developed hepatic steatosis (82). In that study (82), ovariectomized mice on the high fat diet exhibited worse steatosis than sham-operated mice fed the same diet, indicating that although ovarian hormone deficiency may not always lead to increased hepatic TG content, it increases susceptibility to diet-induced impairments in hepatic lipid metabolism. Additionally, the time elapsed since the last meal could possibly contribute to discrepancy among studies. In the present study, mice were fasted for approximately 8 hours as done previously by our group (142) to maximize clearance of intestine-derived chylomicron-TG in the blood. In ovariectomized mice fasted 4-5 hr before sacrifice, no changes in hepatic TG were observed (63).

Furthermore, acute bouts of fasting for 24 and 48 hr prior to sacrifice can attenuate hepatic lipid accumulation induced by weeks of high-fat diet consumption and dramatically reduce hepatic TG synthesis, respectively, compared to non-fasting controls (34, 103). The type of diet and fasting duration therefore can have a dramatic effect upon hepatic TG metabolism and can make comparisons across studies difficult to interpret. In obese humans, level of hepatic but not total visceral fat is associated with impaired insulin sensitivity and elevated VLDL-TG secretion (33). However, we found reduced glucose tolerance and higher fasting blood glucose in OVX vs. SHAM with no differences in VLDL-TG secretion even despite lower hepatic TG, which might suggest that the role of fatty liver in blood glucose dysregulation in menopausal women may be relatively less important than other metabolic impairments. Ovariectomy inhibits both insulin-stimulated suppression of hepatic glucose output (25) and whole-body glucose uptake (110) and so interventions to improve insulin sensitivity after menopause should target both liver and peripheral tissue to maximize effectiveness.

Previously we examined the effects of single bouts of these exercise protocols (CE and HIIE) and showed that in females (with intact ovaries), HIIE produced greater elevations in hepatic TG and PLIN2 than CE on the day of exercise and that on the day after exercise only HIIE lowered VLDL-TG secretion (142). In the present study, indirect calorimetry confirmed that energy expenditure over the duration of HIIE and CE bouts are identical. Therefore differences in the effects of these exercise modalities in response to single exercise sessions (142) and training (present results) are due to intensity rather than total energy expenditure of exercise. Although we previously

showed that an acute bout of HIIE lowered VLDL-TG secretion on the day after exercise (142), in the present study of chronic training, we did not observe any exercise-related changes in VLDL-TG kinetics on the day after the most recent exercise bout even though this bout duration was 50% longer (45 vs. 30 min). Mice were untrained in the previous study (142) and thus the 30 s intervals of running at 30 m/min were likely relatively more intense than after training in the present study. Together, these findings may suggest that the relative intensity of exercise is more important than the training volume for changes in VLDL-TG secretion, and exercise intensity might need to be especially intense in the trained state to alter hepatic lipid kinetics appreciably. Thus when designing HIIE training prescriptions aimed at consistently altering VLDL-TG kinetics, it may be necessary to progressively increase exercise intensity over time to maintain relatively high physiological stress.

Although no effects of training were found for hepatic TG content and VLDL-TG secretion, abundance of the lipid metabolism-regulating enzyme AMPK was increased in OVX and SHAM only with chronic HIIE. Previously, we demonstrated that an acute bout of CE or HIIE did not increase hepatic AMPK content (142), indicating that the increase with HIIE training was a chronic adaptation rather than an acute response to the most recent exercise bout. This is in accord with another study demonstrating increased hepatic AMPK after 12 weeks of continuous exercise training in rats (132). To our knowledge, however, our study is the first to show hepatic AMPK protein expression is increased in an intensity-dependent manner following chronic training. The physiological relevance of increased hepatic AMPK with HIIE training is unclear, but

could possibly be related to enhanced ability for oxidative disposal of FAs. This may be necessary to cope with the habitual increase in adipose FA release into circulation and subsequent delivery to the liver that occurs during training. AMPK can increase FA oxidation by deactivating and activating ACC and malonyl-CoA decarboxylase, respectively, through phosphorylation (113). We did not observe a parallel increase in phosphorylated (activated) AMPK probably because hepatic AMPK is activated during/immediately after exercise (20, 106) but not the following day (142). This may explain the lack of change in phosphorylated ACC. Although HIIE training did not lead to reductions in hepatic TG, the resulting elevation in hepatic AMPK may have protective qualities. AMPK activating agents have hepatic lipid lowering effects (73, 108, 154) and thus the increase in hepatic AMPK with HIIE could possibly reduce susceptibility to fatty liver development induced by high-fat feeding in ovarectiomized mice (82) or other conditions.

Indirect calorimetry indicated that absolute metabolic and lipid oxidation rates were slightly greater in OVX compared to SHAM mice and is consistent with their higher body mass. However, in agreement with other studies in mice (112) and humans (81), relative metabolic rate was lower in OVX vs. SHAM. No differences were found between OVX and SHAM for SPA, and so the observed difference in relative energy expenditure was due to metabolic rather than behavioral perturbations. Post-exercise SPA, however, was measured in the light phase and no differences were found between OVX and SHAM. This is could be related to mice being nocturnal and is consistent with previous work showing ovarectomized mice to be less active during the dark but not

light phase (112). The previous work showed that in the dark phase, ovariectomized mice have both lower energy expenditure and decreased activity relative to sham-operated mice (112), and so the overall lower metabolic rate caused by ovarian hormone deficiency could be due to both metabolic and behavioral disturbances.

In humans, exercise results in elevated energy expenditure (39) and enhanced relative fat oxidation (29, 53, 55) in the post-exercise period. In the present study, indirect calorimetry over 4 hours immediately after exercise showed that exercise bouts did not significantly alter energy expenditure in SHAM or OVX. However, CE and HIIE similarly increased relative fat oxidation (reduced RER) to a modest extent with a trend for a higher increase with HIIE than CE in SHAM and this reduction in RER after exercise is consistent with human studies (29, 53, 55). Despite these increases in relative fat oxidation, no exercise related changes in body composition or hepatic TG were observed. Substrate oxidation rates revealed that the increase in relative fat oxidation with exercise was not attributed to an increase in absolute lipid oxidation but rather to a decreased rate of absolute carbohydrate oxidation, and this was associated with a non-significant reduction in metabolic rate. Thus, although the proportion of fat contributing to energy expenditure was increased, total fat oxidation and metabolic rate were not. Both exercise types also resulted in marked decreases in SPA during the post-exercise period and this may explain the lower absolute carbohydrate oxidation rate and the non-significant reduction in metabolic rate after exercise. It is not known how long after exercise these effects on activity persist, but because the magnitude of the decreases were quite large, it is possible that they continued long enough to compensate for the

activity of exercise. A similar phenomenon has been found using accelerometers worn by in elderly humans in which activity of exercise is compensated for by a decrease in non-exercise activity such that total daily physical activity is not changed (97), although not all human studies agree with this finding (146). Because of the potential decrease in activity after exercise and the lowering of activity following ovarian hormone deficiency (112), interventions for women with menopause and other individuals attempting to lower body fat should encourage both exercise and non-exercise activity.

OVX displayed decreased grip strength and there is some evidence in humans that strength declines with menopause (67). Ovariectomy in mice results in impaired force production in isolated muscle without loss of muscle mass (102) and this agrees with our findings of lowered grip strength despite no change in quadriceps weight in OVX vs. SHAM. Grip strength measurement, which has been used with other models of neuromuscular dysfunction (54, 76, 141), may thus be a relatively easier and quicker alternative for assessing effectiveness of interventions aimed at improving strength in this particular animal model. Because HIIE is somewhat qualitatively similar to resistance exercise (i.e., short periods of high-intensity exertion interspersed with rest periods), and because there is some evidence that HIIE (24) and high-intensity CE (26) could increase muscle fiber cross-sectional area, we expected HIIE to reduce the impairment in strength in OVX. However, we did not observe any exercise related changes. Despite some resemblance to resistance exercise and possible increases in fiber area, high-intensity interval exercise produces similar adaptations in skeletal muscle related to oxidative capacity as traditional continuous exercise (7, 19, 41) and

based on the present work appears not to significantly impact volitional force production. Because HIIE can potentially improve lipid metabolism in skeletal muscle via enhanced oxidative capacity (7, 19, 41) and in liver, possibly through increased hepatic AMPK (present results), it could be combined with resistance exercise as a strategy to improve both the whole-body metabolic and neuromuscular dysfunctions that occur in women with menopause.

In conclusion, we showed that during ovarian hormone deficiency, blood glucose dysregulation, decreased relative energy expenditure, and increased body fat can occur in the absence of fatty liver and that functional strength also declines. Exercise increased hepatic AMPK expression despite compensation for the energy expenditure of exercise through reductions in post-exercise SPA. We discovered different effects of CE and HIIE on hepatic AMPK expression despite bouts being identically matched for energy expenditure, distance, and duration; thus, we revealed another fundamental difference in the physiological responses between these two exercise modalities. Together with our previous work that uncovered unique attributes of the response of hepatic metabolism to single bouts of HIIE (142), we have demonstrated distinct metabolic responses to chronic vs. acute exercise as well as intensity-dependent effects. The effects of chronic exercise training appear to depend upon endurance exercise modality and are different from the responses to single bouts of exercise in the untrained state. The results suggest that it is important to tailor exercise prescription to specific populations to achieve desired metabolic alterations. Previous work has detailed sexual dimorphism in the response to exercise (52, 53, 55). Additional efforts

are needed to carefully characterize the differences between females with and without ovarian function to inform ideal intervention design that targets specific metabolic impairments that occur during ovarian hormone deficiency.

ACKNOWLEDGEMENTS

The authors thank Keith Anacker, Kristen Rizkalla, Taylor R. McConnell, Daphne Bienkiewicz, and Zunaira Khan for conducting exercise sessions and assisting with experiments. The authors also thank Dr. Judith Storch for use of the EchoMRI and Oxymax systems, Dr. Angela Gadja for advice regarding Oxymax system use, and Dr. Dawn Brasaemle for providing PLIN2 antibody.

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GRANTS

This work was supported by the Division of Life Sciences at Rutgers University and by American Diabetes Association grant # 7-13-JF-27-BR to G.C. Henderson.

DISCLOSURES

The authors declare no conflicts of interest.

Table 3-1. VLDL-TG kinetics, plasma metabolite concentrations, and hepatic TG content.

	SHAM			OVX		
	CON	CE	HIIE	CON	CE	HIIE
VLDL-TG secretion (mg/dL/min)	5.01±0.42	5.43±0.31	4.78±0.18	4.87±0.31	5.21±0.21	5.53±0.28
VLDL-TG secretion (mg/min/kg BW)	5.51±0.46	5.97±0.34	5.25±0.20	5.36±0.34	5.73±0.23	6.08±0.31
Plasma [TG] (mg/dL)	60±10	68±5	54±2	54±7	73±10	54±4
Plasma [glycerol] (mM)	0.49±0.12	0.40±0.08	0.25±0.03	0.25±0.03	0.34±0.07	0.23±0.03
Blood [glucose] (mg/dL)^	135±8	129±6	120±4	148±5	138±8	137±6
Plasma [insulin] (ng/mL)‡	1.19±0.35	0.52±0.03$^{\delta}$	0.48±0.07$^{\delta}$	0.81±0.21	0.70±0.14$^{\Delta}$	1.42±0.26
Hepatic [TG] % wet wt (mg/mg tissue)$^{\&}$	1.23±0.21	1.54±0.28	1.33±0.23	0.99±0.17	0.98±0.19	0.73±0.08

Values are means ± SE. n=7-9 per group for VLDL-TG secretion and 5-6 for plasma and hepatic concentrations. BW, bodyweight. ^Significantly greater in OVX vs. SHAM (main effect of surgery, $p<0.05$). $^{\&}$Significantly lower in OVX vs. SHAM (main effect of surgery, $p<0.01$). ‡Surgery-by-training interaction, $p<0.05$. $^{\delta}$Significantly different from SHAM CON, $p<0.05$. $^{\Delta}$Significantly different from OVX HIIE, $p<0.05$.

Figure 3-1

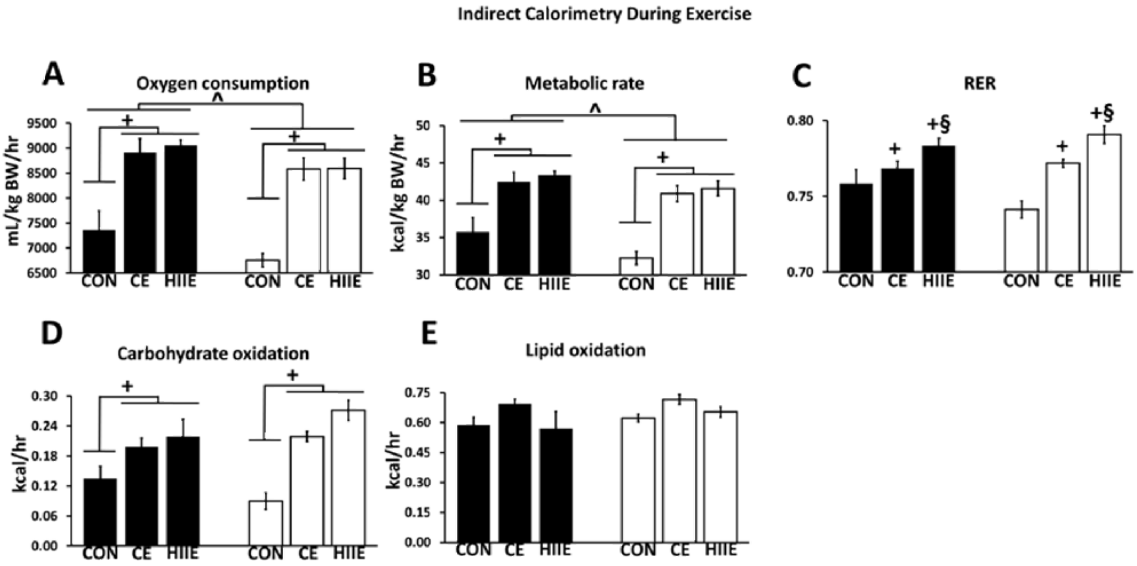

Figure 3-1. Treadmill indirect calorimetry. Values are means ± SE. n=6-8 per group. SHAM, black bars. OVX, open bars. A: Mean oxygen consumption (VO_2) normalized to bodyweight (BW). ^Main effect of surgery, $p<0.05$. Main effect of training, $p<0.0001$. $^{+}$CE and HIIE significantly different from CON ($p<0.05$) but not from each other. No surgery-by-training interaction. B: Mean metabolic rate normalized to BW. ^Main effect of surgery, $p<0.05$. Main effect of training, $p<0.0001$. $^{+}$CE and HIIE significantly different from CON ($p<0.05$) but not from each other. No surgery-by-training interaction. C: Mean respiratory exchange ratio (RER). Main effect of training, $p<0.0001$. $^{+}$Significantly different from CON, $p<0.05$. §Significantly different from CE, $p<0.05$. No main effect of surgery or surgery-by-training interaction. D: Mean carbohydrate oxidation rate. Main effect of training, $p<0.0001$. $^{+}$CE and HIIE significantly different from CON ($p<0.05$) but not from each other. No surgery-by-training interaction. E: Mean lipid oxidation rate. No main effects or interaction.

Figure 3-2

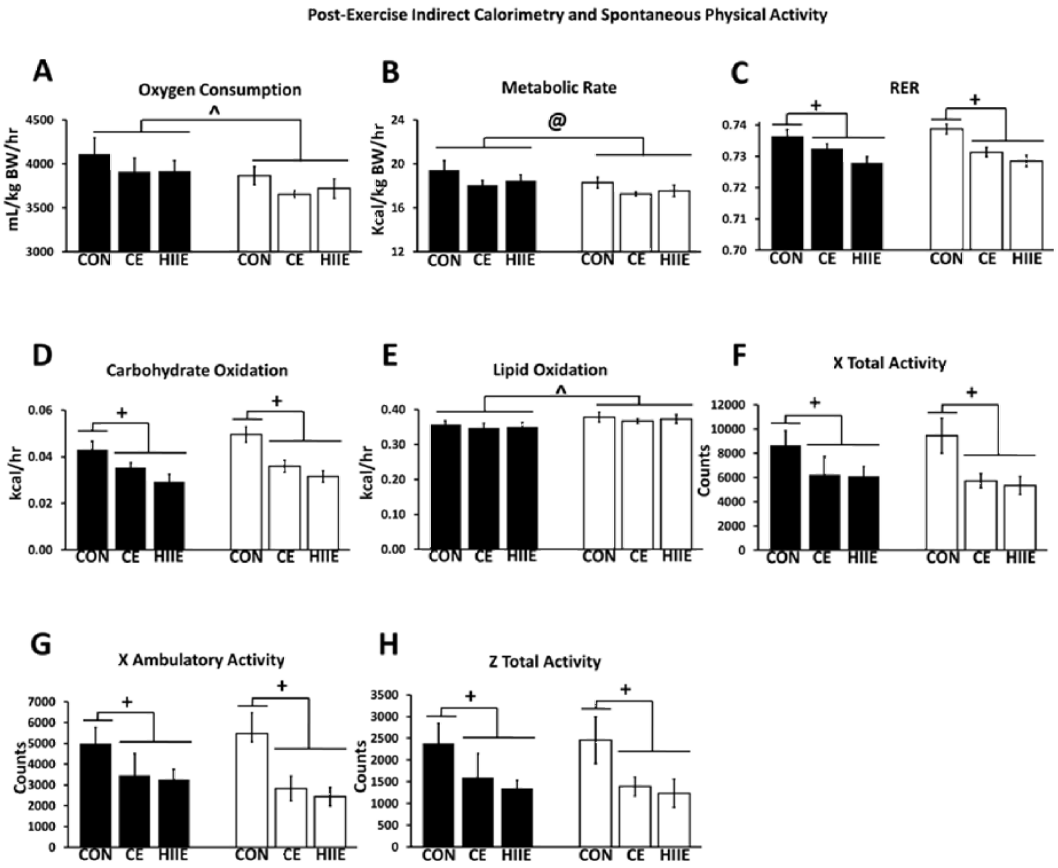

Figure 3-2. Post-Exercise indirect calorimetry and spontaneous physical activity. Values are means ± SE. n=7-8 per group. SHAM, black bars. OVX, open bars. A: Mean oxygen consumption (VO_2) normalized to bodyweight (BW). ^Main effect of surgery, $p<0.05$. No main effect of training and no surgery-by-training interaction. B: Mean metabolic rate normalized to BW. @Main effect of surgery, $p=0.05$. No main effect of training and no surgery-by-training interaction. C: Mean respiratory exchange ratio (RER). Main effect of training, $p<0.0001$. +CE and HIIE significantly different from CON ($p<0.05$) but not from each other. No main effect of surgery or surgery-by-training interaction. D: Mean carbohydrate oxidation rate. Main effect of training, $p<0.0001$. +CE and HIIE significantly different from CON ($p<0.05$) but not from each other. No

surgery-by-training interaction. E: Mean lipid oxidation rate. ^Main effect of surgery, $p<0.05$. No main effect of training and no surgery-by-training interaction. F: X total activity. Main effect of training, $p<0.01$. $^{+}$CE and HIIE significantly different from CON ($p<0.05$) but not from each other. No main effect of surgery or surgery-by-training interaction. G: X ambulatory activity. Main effect of training, $p<0.01$. $^{+}$CE and HIIE significantly different from CON ($p<0.05$) but not from each other. No main effect of surgery or surgery-by-training interaction. H: Z total activity. Main effect of training, $p<0.05$. $^{+}$CE and HIIE significantly different from CON ($p<0.05$) but not from each other. No main effect of surgery or surgery-by-training interaction.

Figure 3-3

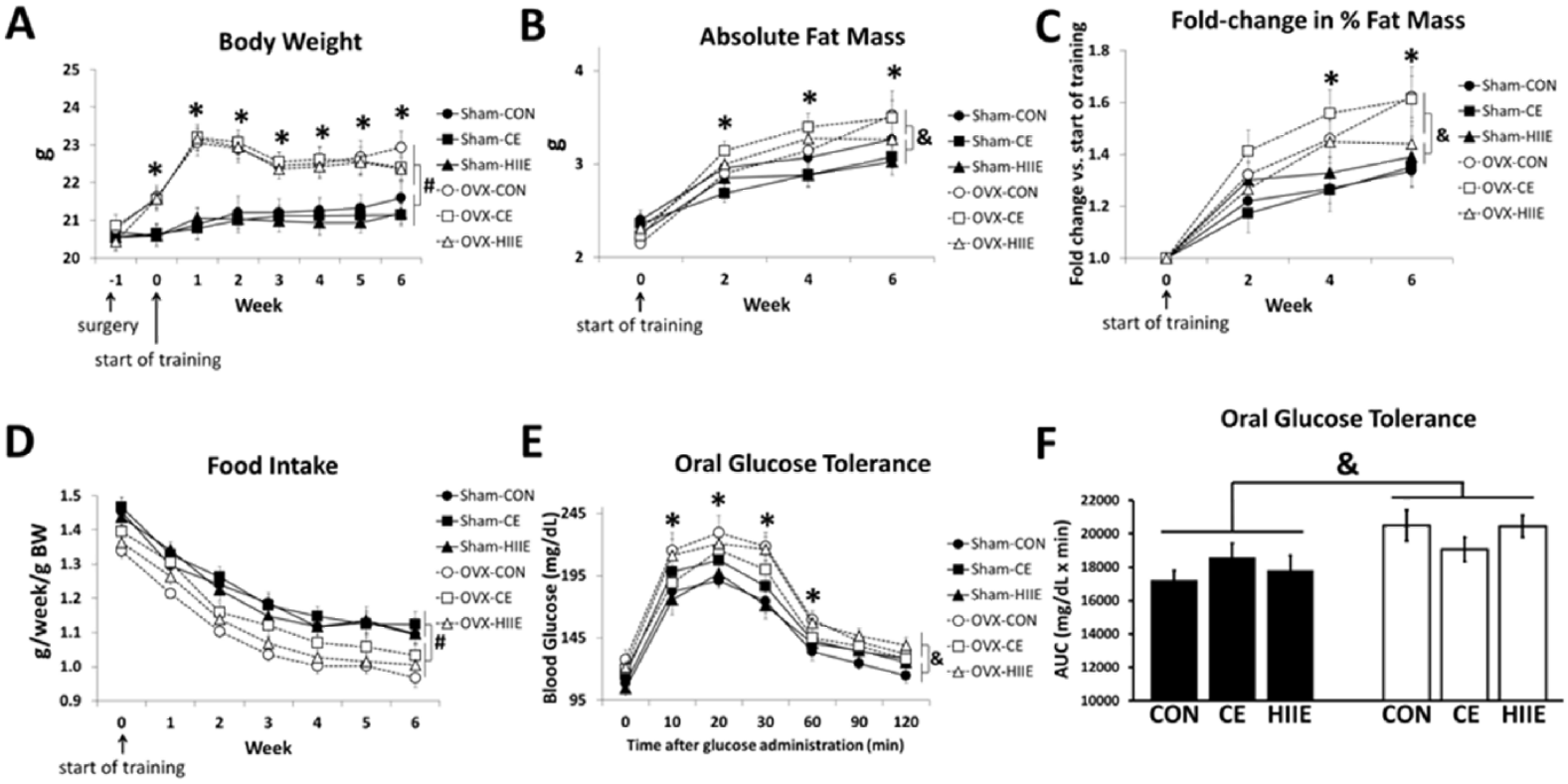
A
Body Weight
g
Week
surgery
start of training
Sham-CON
Sham-CE
Sham-HIIE
OVX-CON
OVX-CE
OVX-HIIE
#
B
Absolute Fat Mass
g
Week
start of training
&
C
Fold-change in % Fat Mass
Fold change vs. start of training
Week
start of training
&
D
Food Intake
g/week/g BW
Week
start of training
#
E
Oral Glucose Tolerance
Blood Glucose (mg/dL)
Time after glucose administration (min)
&
F
Oral Glucose Tolerance
&
AUC (mg/dL x min)
CON
CE
HIIE
CON
CE
HIIE

Figure 3-3. Body composition, food intake, and oral glucose tolerance. Values are means ± SE. n=13-15 per group for body composition and food intake measurements. n=9-10 per group for oral glucose tolerance tests. A: Body weight (BW). #Main effect of surgery, $p<0.0001$. Main effect of time, $p<0.0001$. Surgery-by-time interaction, $p<0.0001$. *Significantly different between OVX and SHAM at individual time point, $p<0.05$. No main effect of training and no other interactions. B: Absolute fat mass. &Main effect of surgery, $p<0.01$. Main effect of time, $p<0.0001$. Surgery-by-time interaction, $p<0.01$. *Significantly different between OVX and SHAM at individual time point, $p<0.05$. No main effect of training and no other interactions. C: Fold-change in percent fat mass. &Main effect of surgery, $p<0.01$. Main effect of time, $p<0.0001$. Surgery-by-time interaction, $p<0.001$. *Significantly different between OVX and SHAM at individual time point, $p<0.05$. No main effect of training and no other interactions. D: Food intake normalized to BW. #Main effect of surgery, $p<0.0001$. Main effect of time, $p<0.0001$. No main effect of training and no interactions. E: Oral glucose tolerance test. &Main effect of surgery, $p<0.01$. Main effect of time, $p<0.01$. Surgery-by-time interaction, $p<0.01$. *Significantly different between OVX and SHAM at individual time point, $p<0.05$. No main effect of training and no other interactions. F: Oral glucose tolerance test area under the curve (AUC). &Main effect of surgery, $p<0.01$. No main effect of training and no surgery-by-training interaction.

Figure 3-4

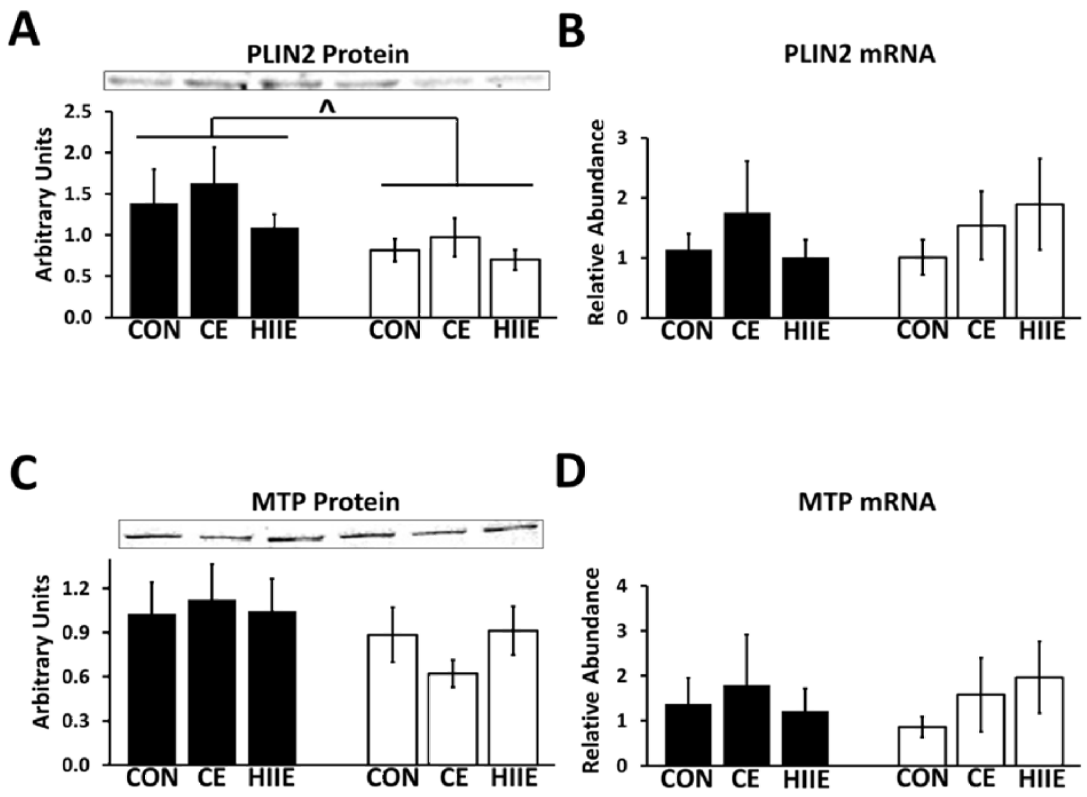

Figure 3-4. Hepatic PLIN2 and MTP expression. Values are means ± SE. n=5-6 per group. SHAM, black bars. OVX, open bars. A: PLIN2 protein. ^Main effect of surgery, $p<0.05$. No main effect of training or surgery-by-training interaction. B: PLIN2 mRNA. No main effects or interaction. C-D: MTP protein and mRNA, respectively. No main effects or interactions.

Figure 3-5

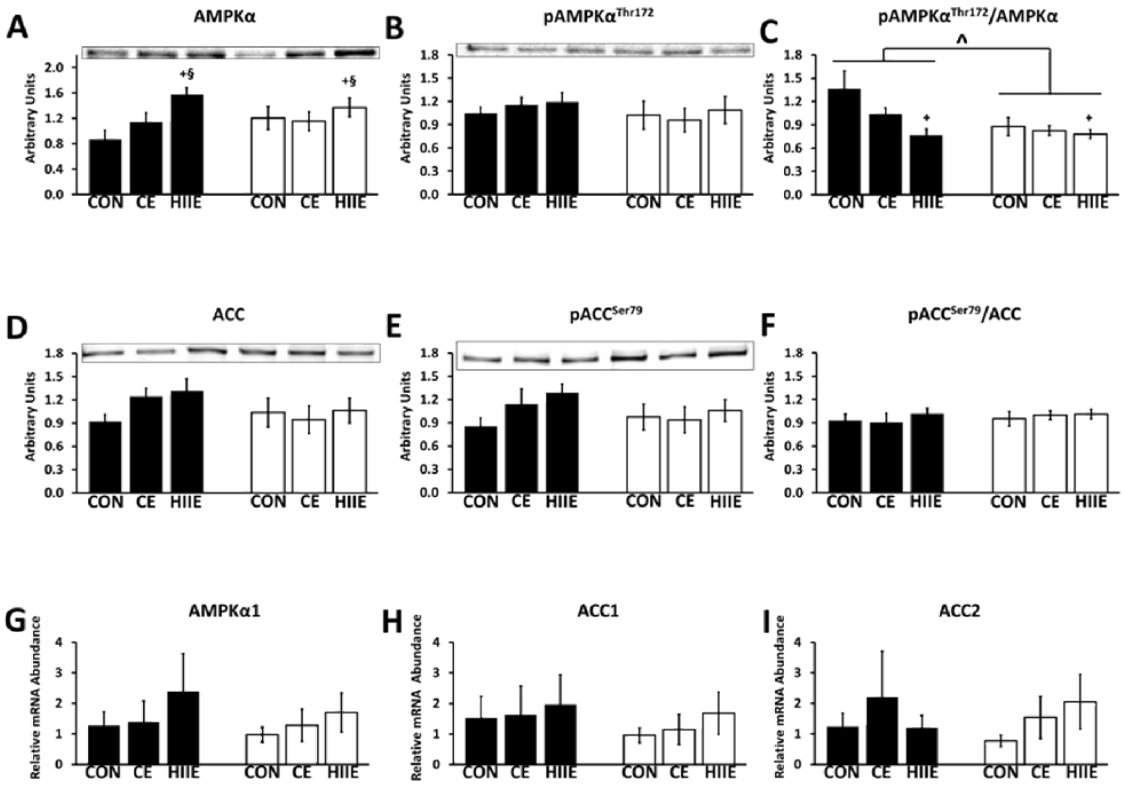

Figure 3-5. Hepatic AMPK signaling and related protein and gene expression. Values are means ± SE. n=5-6 per group. SHAM, black bars. OVX, open bars. A: Total AMPKα. Main effect of training, $p<0.05$. $^{+}$Significantly different from CON, $p<0.05$. §Significantly different from CE, $p<0.05$. No main effect of surgery or surgery-by-training interaction. B: Phosphorylated AMPKα^{Thr172}. No main effects or interaction. C: Ratio of phosphorylated AMPKα^{Thr172} to total AMPKα. ^Main effect of surgery, $p<0.05$. Main effect of training, $p<0.05$. $^{+}$Significantly different from CON, $p<0.05$. No surgery-by-training interaction. D-F: Total ACC, phosphorylated ACC^{Ser79}, and ratio of phosphorylated ACC^{Ser79} to total ACC, respectively. No main effects or interactions. G-I: mRNA expression of AMPKα1, ACC1, and ACC2, respectively. No main effects or interactions.

Figure 3-6

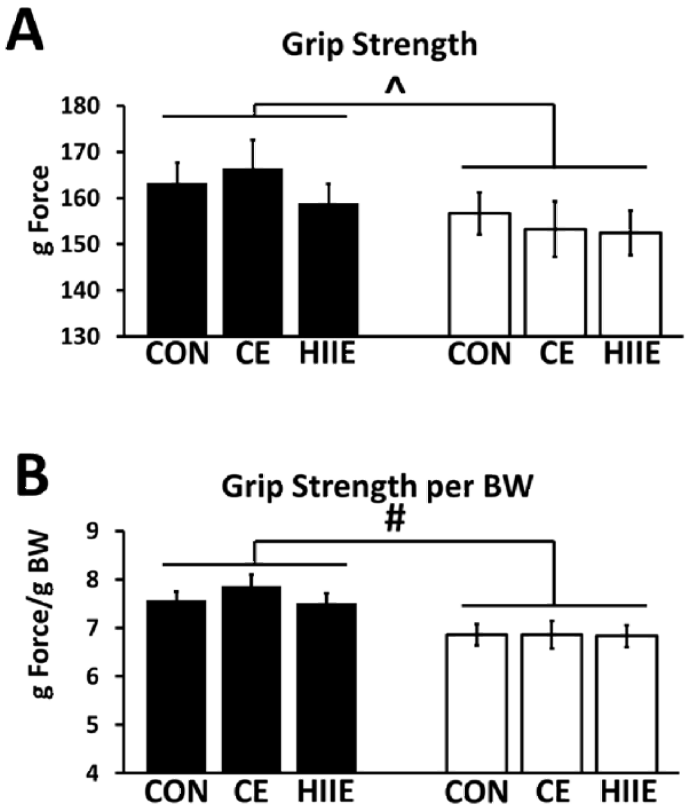

Figure 3-6. Grip strength. Values are means ± SE. n=13-15 per group. SHAM, black bars. OVX, open bars. A: Absolute strength. ^Main effect of surgery, $p<0.05$. No main effect of training or surgery-by-training interaction. B: Strength normalized to bodyweight. #Main effect of surgery, $p<0.0001$. No main effect of training or surgery-by-training interaction.

Chapter 4: Discussion and Conclusions

Elevated plasma TG, circulating in the fasted state primarily as VLDL-TG, and excess hepatic TG accumulation are associated with increased coronary heart disease risk (4) and insulin resistance (33, 66, 118), respectively. Thus, the development of therapeutic interventions targeting these aspects of lipid metabolism is an attractive strategy for reducing risk of chronic diseases. In Aim 1, we examined the impact of single bouts of moderate-intensity CE versus HIIE on hepatic TG metabolism and secretion in female and male mice. Our novel findings regarding responses to exercise were as follows. Both CE and HIIE transiently increased hepatic TG concentration and in females, the increase was greater with HIIE. Hepatic protein abundance of the LD-coating protein PLIN2 was also transiently increased by exercise with a greater increase with HIIE in both sexes. Increases in PLIN2 protein with exercise appeared mediated by increased gene transcription. In turn, increased PLIN2 can prevent hydrolysis of lipid droplets by blocking access of cytosolic lipases to substrate lipids (14, 77). Thus, it seems that hepatic TG is increased in response to exercise in an intensity-dependent manner through transcriptional regulation of PLIN2 likely followed by a reduction in lipolysis via increased PLIN2 protein (Figure 4-1). Further, reduced VLDL-TG secretion rate the day after exercise occurred only in females with HIIE in association with enhanced hepatic TG storage, and this finding could potentially provide clues about molecular mechanisms behind alterations in VLDL-TG kinetics in women in response to exercise (9). Importantly, these differences between CE and HIIE occurred even despite bouts being identical in total distance ran, bout duration, and energy expenditure (Aim 3) and without differences between HIIE and CE for ad libitum food intake. These

results show that acute exercise alters hepatic TG metabolism and secretion in both intensity-dependent and sex-specific manners. We also found greater basal VLDL-TG secretion rate in females, a sexual dimorphism also displayed by humans (88, 98). Thus, the common laboratory animal used provides a model for studying molecular mechanisms underpinning sex-based differences in VLDL-TG secretion in humans and our findings suggest that the higher secretion rate in females may be related to greater abundances of hepatic TG, AMPK, ACC, and MTP (Figure 4-1). Together, these findings may have implications for designing exercise interventions aimed at influencing specific aspects of hepatic TG metabolism in each sex.

Figure 4-1

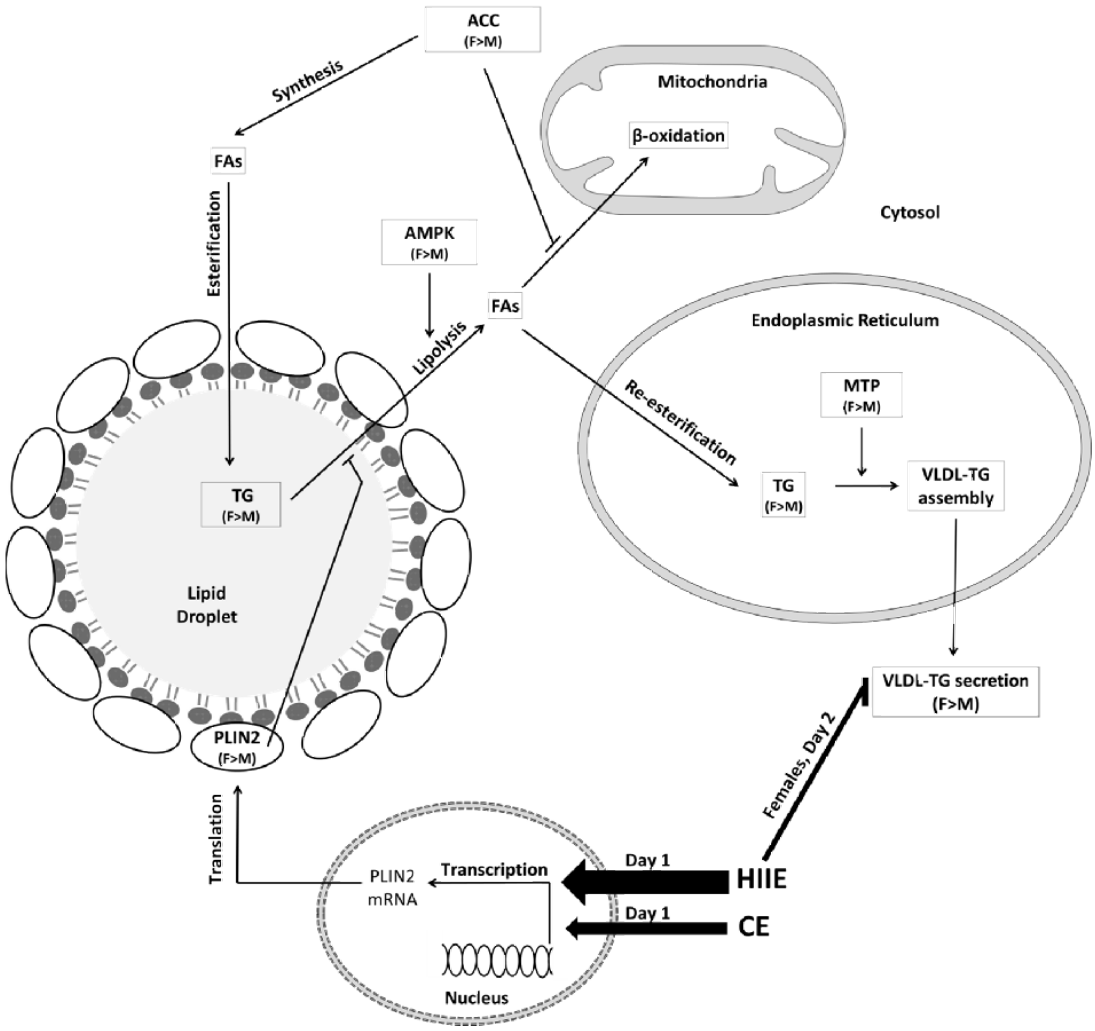

Figure 4-1. Potential mechanisms for effects of acute exercise on hepatic TG metabolism and sexual dimorphism in basal VLDL-TG secretion rate. (F>M) indicates significantly greater in females than males. Exercise transiently increases hepatic TG concentration on Day 1 likely through transcriptional regulation of PLIN2 expression, with significant increases in PLIN2 mRNA only with HIIE. On Day 2, HIIE in females results in decreased VLDL-TG secretion rate (no effect of CE and no effect of exercise in males) possibly through inhibition of lipid droplet TG lipolysis, as evidenced

by a trend for ~50% increase in hepatic TG concentration. Under basal sedentary conditions, females exhibit higher VLDL-TG secretion rate than males. ACC catalyzes the synthesis of malonyl-CoA, a substrate for FA synthesis and an inhibitor of FA transport into the mitochondria for β-oxidation (126). Higher abundance of ACC may enhance the formation and partitioning of FAs toward esterification into TG resulting in higher TG content in lipid droplets. FAs from lipid droplet TG may be released by AMPK-stimulated lipolysis (50) for transport into the endoplasmic reticulum for re-esterification, potentially resulting in higher TG availability (turnover) for incorporation into VLDL by MTP and as a result, greater VLDL-TG secretion.

Menopause is associated with numerous adverse outcomes including insulin resistance (74, 85), increased adiposity (40, 58, 70, 81, 85, 129, 134), and declines in muscular strength (67). Exercise training is an attractive approach for helping to prevent these negative health effects, however, the relative efficacies of different exercise modalities is largely unknown. In Aim 1, we discovered HIIE to be more effective than CE at influencing lipid metabolism and thus decided to compare the effects of 6 weeks of training with these two exercise modalities on hepatic TG metabolism, glucose tolerance, body composition, and strength in ovariectomized and sham-operated mice in Aim 2. Ovarian hormone deficiency impaired glucose tolerance, elevated fasting blood glucose, and increased adiposity despite lowering hepatic TG, as well as lowered strength, and these outcomes were not affected by training. That these metabolic impairments were present in OVX in the absence of increased hepatic TG content could possibly suggest that the role of fatty liver in dysregulations associated with menopause may not be relatively as important as other metabolic disturbances. Surprisingly, training did not affect body fat, hepatic TG, and glucose tolerance in both OVX and SHAM and this could likely be related to reduced post-exercise SPA. It appeared that the activity of exercise was compensated for by reductions in activity afterwards, and this emphasizes that targeting non-exercise activity may be equally as important as increasing actual exercise in both postmenopausal women and other populations.

In contrast with acute exercise responses to these exercise modalities found in Aim 1 (142), chronic HIIE in Aim 2 led to increased hepatic AMPK protein expression.

Interestingly, another difference between a single session of HIIE (142) vs chronic HIIE is that the acute reduction in VLDL-TG secretion after exercise is not maintained after 6 weeks of training even despite longer exercise bout duration. These findings suggest that relative intensity of exercise is more important than the training volume for changes in VLDL-TG secretion to manifest, and that exercise intensity might need to be especially intense in the trained state to alter hepatic lipid kinetics appreciably. Collectively, our results reveal distinct responses to acute vs. chronic exercise as well as intensity-dependent and sex-specific effects related to hepatic lipid metabolism (summarized in Table 4-1). These findings have implications for the importance of tailoring exercise prescription to specific populations and to desired metabolic alterations. Below, we discuss potential future studies that could be conducted to understand possible physiological significances of our findings and underlying molecular mechanisms, as well as to address other interesting questions raised by our work.

Table 4-1. Distinct responses to acute vs. chronic execise plus intensity-dependent and sex-specific effects.

Outcome	Single exercise bout [Aim 1 (142)]	Chronic training (Aim 2)
Hepatic TG concentration	Increased in response to exercise with a greater increase with HIIE in females.	No effect in SHAM or OVX.
Hepatic PLIN2 expression	Increased in response to exercise with a greater increase with HIIE in both sexes.	No effect in SHAM or OVX.
VLDL-TG secretion rate	Decreased only with HIIE in females. No effect of exercise in males.	No effect in SHAM or OVX.
Total AMPK abundance	No effect.	Increased only with HIIE in SHAM and OVX.

Future Directions

In Aim 1, we found that exercise transiently elevated hepatic TG content in an intensity-dependent manner likely through increased transcription of PLIN2 (142). The physiological significance of this is currently unknown. It could be that it promotes a period of enhanced hepatic insulin sensitivity. Acute bouts of continuous exercise prevent lipid-induced whole-body insulin resistance by lowering concentrations of lipotoxic metabolites in skeletal muscle, partially by promoting storage of FAs as biologically inert TG (114). In addition, PLIN2 overexpression in muscle also increases insulin sensitivity in parallel with greater IMTG (12). It is plausible that a similar phenomenon in the liver in response to exercise could occur because certain alterations in lipid metabolism with exercise are common between liver and muscle (106). Studies in the future using hyperinsulinemic-euglycemic clamp and glucose tracer methodology to assess hepatic insulin-sensitivity as well as measuring lipotoxic metabolite concentrations during the period of increased hepatic TG after exercise could test this hypothesis.

How PLIN2 transcription is increased by exercise and to a greater extent with HIIE than CE is also not known, but is likely related to PPARα activation. PPARα is a transcriptional regulator of PLIN2 (27) that is activated by FFAs (38). During exercise, FFAs from adipose lipolysis are released into the blood, which would increase FFA delivery to the liver and in turn possibly activate PPARα. In rodents, the elevated plasma FFA during exercise subsides within 15 min (20) to 3 hr (60) post-exercise. Thus, similar exercise studies in the future comparing CE vs. HIIE in which blood is collected

immediately after exercise (rather than 3 hr post-exercise as in Aim 1) could be used to determine whether the pattern of plasma FFA concentration correlates with our observations of increased hepatic TG. Measurement of plasma catecholamines, growth hormone, and cortisol could also be performed as they are also increased in an exercise intensity-dependent manner (35, 57).

Our findings of suppressed VLDL-TG secretion rate with exercise in females is in agreement with findings in humans (9) and appears related to enhanced hepatic TG storage. Future studies are needed to elucidate the mechanisms behind this relationship. The trend for increased hepatic TG associated with the lower VLDL-TG secretion rate may suggest decreased hydrolysis of TG in LDs, which could reduce FA availability for re-esterification into TG for incorporation into VLDL particles. This could be potentially due to altered LD morphology, such as increased LD size and consequently smaller surface area available for lipases to access, and this could be confirmed by histological analysis. Alternatively, the reduction in VLDL-TG secretion and increase in hepatic TG content, could be attributed to decreased abundance of apoB-100 protein, which is required for assembly and secretion of VLDL particles (151).

In Aim 2, we found SPA to be lowered by exercise during the 4 hr recovery period, but it is unknown how long this effect would persist. SPA was not our primary outcome of interest, and for logistical purposes, was measured only during the light phase. It is possible that the differences in SPA after CE and HIIE compared to sedentary controls were more pronounced in the dark phase when mice are more

active, and this remains to be determined. Additionally, it could be that the reductions in SPA after exercise would have been smaller if assessed towards the end of the training period, when exercise bouts were likely relatively less intense. Also, how hepatic AMPK is increased in an intensity-dependent manner with chronic exercise is unclear, but could be attributed to greater adrenergic stimulation with HIIE (96, 107). Experiments using chronic catecholamine administration could be used to test this idea. As well, the physiological importance of the increase in hepatic AMPK with HIIE is unknown. Perhaps HIIE could reduce susceptibility of ovariectomized mice to high-fat diet-induced fatty liver (82) since AMPK activation can enhance fatty acid oxidation (109) and AMPK activating drugs have lipid lowering effects in the liver (73, 108, 154). Lastly, the role of other sex hormones besides estrogen in the dysregulation of hepatic and whole-body lipid metabolism during ovarian hormone deficiency has not been extensively studied. The importance of estrogen has been demonstrated through its administration to ovariectomized animals (5, 48), and future studies could examine the effects progesterone and testosterone administration.

In conclusion, we discovered intensity-dependent and sex-specific effects in hepatic TG metabolism after acute exercise in Aim 1. By studying hepatic TG metabolism in response to chronic exercise in Aim 2, we found that the effects of acute exercise are not maintained after training and that relatively high exercise intensity is required for certain training-induced adaptations to occur. Our results from Aim 3 revealed that exercise activity is potentially compensated for by reductions in non-exercise activity. Collectively, our findings suggest that in order to maximize the

effectiveness of exercise, it is important to progressively increase exercise intensity during training and to promote non-exercise physical activity between exercise sessions.

Acknowledgement of Previous Publications

Tuazon, M.A., McConnell, T.R., Wilson, G.J., Anthony, T.G., and Henderson, G.C. (2015) Intensity-dependent and sex-specific alterations in hepatic triglyceride metabolism in mice following acute exercise. *Journal of Applied Physiology*. 118, 61-70.

Chapter 2 was previously published in the Journal of Applied Physiology in 2015. Marc A. Tuazon performed the research and writing with assistance from Taylor R. McConnell (undergraduate), Gabriel J. Wilson (postdoctoral fellow), and Tracy G. Anthony (department faculty and committee member), under the guidance of Gregory C. Henderson (corresponding author and former program faculty member).

Appendix

Abstracts of other peer-reviewed publications

Tuazon, M.A. and Henderson G.C. (2012). Fatty acid profile of skeletal muscle phospholipid is altered in mdx mice and is predictive of disease markers. Metabolism. 61:801-11.

Author Contributions: M.A.T. contributed to study design, performed laboratory analyses, analyzed results, interpreted findings, and wrote the manuscript. G.C.H. conceived of and designed the experiment, performed laboratory analyses, analyzed results, interpreted findings, and wrote the manuscript.

Abstract

The mdx mouse is a model for Duchenne muscular dystrophy. The fatty acid (FA) composition in dystrophic muscle could potentially impact the disease severity. We tested FA profiles in skeletal muscle phospholipid (PL) and triglyceride in mdx and control (con) mice to assess associations with disease state as well as correlations with grip strength (which is lower in mdx) and serum creatine kinase (CK, which is elevated in mdx). Compared with con, mdx PL contained less docosahexaenoic acid ($P < .001$) and more linoleic acid ($P = .001$). Docosahexaenoic acid contents did not correlate with strength or serum CK. Linoleic acid content in PL was positively correlated with CK in mdx ($P < .05$) but not con. α-Linolenic acid content in PL was positively correlated with strength in mdx ($P < .05$) but not con. The FA profile in triglyceride showed less difference between groups and far less predictive ability for disease markers. We

conclude that profiling the FA composition of tissue lipids (particularly PL) can be a useful strategy for generating novel biomarkers and potential therapeutic targets in muscle diseases and likely other pathological conditions as well. Specifically, the present results have indicated potential benefits of raising content of particular n-3 FAs (especially α-linolenic acid) and reducing content of particular n-6 FAs (linoleic acid) in PL of dystrophic muscle.

Tuazon, M.A. and Henderson G.C. (2013). Fatty acid profile of cardiac muscle phospholipid and triacylglycerol in MDX mice and C57BL/10ScSnJ controls. Lipids. 48:849-51.

Author Contributions: M.A.T. contributed to study design, performed laboratory analyses, analyzed results, interpreted findings, and wrote the manuscript. G.C.H. conceived of and designed the experiment, performed laboratory analyses, analyzed results, interpreted findings, and wrote the manuscript.

Abstract

The mdx mouse is a model for Duchenne muscular dystrophy (DMD), a debilitating disease affecting striated muscle. It is established that the fatty acid (FA) composition of skeletal muscle phospholipid (PL) is altered in mdx mice, but it is not known if cardiac muscle is similarly affected by dystrophin-deficiency. We tested FA profiles in PL and

triacylglycerol (TAG) in cardiac muscle of 12-week old mdx and control (con) mice. Of 22 different FA, similar to our previous finding for skeletal muscle, the most abundant FA in heart PL were palmitic, stearic, cis-vaccenic, linoleic, and docosahexaenoic acid, while for TAG the most abundant FA were palmitic, oleic, cis-vaccenic, and linoleic acid. In comparing mdx and con, no significant group differences were detected for any FA in PL or TAG. Thus, unlike skeletal muscle, FA composition in cardiac muscle PL is not different between mdx and con at the age studied. The results can be understood in the context of tissue-specific disease severity in mdx mice, as pathology is quite modest in cardiac compared with skeletal muscle.

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