

**INFLUENCE OF HABITUAL PHYSICAL ACTIVITY AND ACUTE EXERCISE ON
LIPID METABOLISM AND INSULIN ACTION**

Rachael Katharine Nelson

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Dissertation committee:

Professor Jeffrey F. Horowitz, Chair
Assistant Professor Peter F. Bodary
Adjunct Professor Charles Burant
Professor Gregory Cartee

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Abstract

Influence of habitual physical activity and acute exercise on lipid metabolism and insulin action

by

Rachael Katharine Nelson

Although a physically active lifestyle is clearly an important contributor to long-term health and disease prevention, it remains unclear how much of these health benefits stem from the acute effects of the most recent session(s) of exercise, and how much can be attributed to physiologic adaptations accrued as a result of habitual active. Importantly, the time course for adaptations that occur in the early stages of adopting a habitually active lifestyle is not clearly understood. Therefore, my dissertation projects were designed to examine key metabolic adaptations that occur in response to a more physically active lifestyle in obese adults who are at risk for developing chronic metabolic diseases, including type 2 diabetes. The major findings of my dissertation studies included: In STUDY#1, using data from the National Health and Nutrition Examination Survey (NHANES), we found that insulin sensitivity was significantly associated with the amount of time participants were engaged in physical activity behaviors ($P=0.03$), but not with their level of cardiorespiratory fitness. In STUDY #2, contrary to our hypothesis, Insulin Sensitivity Index (ISI) was significantly higher in a group of overweight adults who exercise regularly compared with a well-matched cohort of sedentary overweight adults, even though the regular exercisers abstained from exercise for 3 days. In this study we also found that a single session of moderate-intensity exercise was sufficient to increase ISI the day after exercise by nearly 25% in the previously sedentary overweight adults – but not in the regular exercisers, eliminating the difference in ISI between the groups that was evident at baseline. In STUDY #3, although ISI was not improved in the hours after the first low-intensity exercise training session, after 2 weeks of training, post-exercise ISI was enhanced. We also discovered that a single session of exercise at 50% $VO_2\text{max}$ (i.e., maximal oxygen uptake) for 40 min may be below a “threshold” for exercise

stimulus required to improve insulin sensitivity into the next day in obese adults. These studies provide important new information for optimizing the design of lifestyle interventions specifically aimed at enhancing metabolic health in overweight and obese individuals.

CHAPTER 1

Statement of the problem

Two-thirds of the U.S. population is classified as overweight or obese (10). Along with this alarming prevalence of obesity, there is also a very high incidence of obesity-related diseases, such as Type 2 Diabetes Mellitus (T2DM) (12, 13). Over twenty-five million people in the United States have T2DM and there are nearly two million new cases reported each year (1). In addition, it is estimated that 35% of U.S. adults have impaired fasting glucose, and are at increased risk for developing T2DM (1). Unfortunately, T2DM contributes to other life threatening diseases including cardiovascular and renal disease. In addition to the aforementioned health consequences, diabetes also contributes to the growing financial burden of health care costs at a rate well over \$100 billion per year (1). Therefore, the prevention and treatment of T2DM is a serious public health concern.

Insulin resistance, defined as a subnormal response to a physiologic dose of insulin, is a major underlying symptom of T2DM, and it is also linked to the development of many other chronic diseases (9, 11). Although losing only 5-10% of initial body weight can improve insulin resistance (15), even this very modest weight loss can be very difficult to both achieve and sustain (6). Importantly, increased physical activity can improve insulin resistance and impart important metabolic health benefits even without weight loss (8). Because regular physical activity can also enhance maximal oxygen uptake (i.e., VO_{2max}), a measure of cardiorespiratory fitness, some of the physiologic adaptations underlying changes in VO_{2max} with exercise (e.g., mitochondrial biogenesis) have been credited with much of the exercise-induced improvements in metabolic health. Consequently most current exercise recommendations are designed with the goal to improve “fitness”. However, mounting evidence indicates that many health benefits of exercise are not directly linked with improvements in VO_{2max} (3, 8, 14), and it is quite possible that training programs designed to improve cardiorespiratory fitness may not be optimally designed for improving metabolic health. This is particularly relevant because the more vigorous exercise

intensity required to improve VO_{2max} has been associated with a greater incidence of injuries and participant discomfort, leading to poor exercise adherence (5).

Epidemiological evidence clearly indicates that even a rather modest increase in regular physical activity behavior can profoundly reduce disease risk (2, 16). However, this level of activity is often found to be insufficient to induce meaningful improvements in classic measures of cardiorespiratory fitness (e.g., maximal oxygen uptake; VO_{2max}) (7). Conversely, regularly performed vigorous exercise (which is known to induce the most robust improvements in VO_{2max}) does not appear to result in further reduction in disease risk beyond that found with light and moderate intensity exercise (2, 16). Moreover, profound improvements in markers of cardiovascular and metabolic health, including insulin resistance are apparent in the several hours after a single session of exercise (4), which is clearly not enough of an exercise stimulus to evoke physiologic adaptations to improve aerobic capacity (i.e., VO_{2max}). Therefore, although a physically active lifestyle is clearly an important contributor to long-term health and disease prevention, it remains unclear how much of these health benefits stem from the acute effects of the most recent session(s) of exercise, and how much can be attributed to physiologic adaptations accrued as a result of exercising regularly. Moreover, the time course for adaptations that occur in the early stages of adopting a habitually active lifestyle are not clearly understood.

The studies outlined in my dissertation examined:

- 1) the influence of habitual physical activity behavior and cardiorespiratory fitness on important risk factors for cardiovascular and metabolic disease [**PROJECTS 1 and 2**].
- 2) the effect of a single session of mild exercise on key factors associated with cardiovascular and metabolic disease in overweight and obese adults [**PROJECTS 2 and 3**].
- 3) progressive adaptations in insulin resistance, skeletal muscle lipid metabolism, and factors regulating these processes as previously sedentary obese adults begin a modest exercise program (i.e., after 1 day, 2 weeks, and 3 months of a low-intensity exercise training program, and again after 3 days without exercise) [**PROJECT 3**]

The overall objective of my dissertation projects was to examine key metabolic responses and adaptations to acute and habitual exercise on factors underlying cardiometabolic disease risk. These findings have important implications for designing lifestyle interventions specifically aimed at enhancing metabolic health in overweight and obese individuals.

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CHAPTER 2

Review of literature

Obesity is now one of the leading public health concerns worldwide. It is estimated that 1.5 billion adults around the world can be classified as overweight and another 500 million as obese (64). The United States has the highest obesity prevalence in the world, with two-thirds of the adult population classified as overweight or obese (26) and obesity rates are continuing to rise (29). Clearly, obesity has a profound impact on disease risk (48) and mortality (17). Life expectancy is thought to be reduced by 4-10 years in overweight and obese compared with non-obese individuals (17). The increased mortality rates may be explained by increased incidence of cardiovascular disease (67), certain types of cancer (70), and type 2 diabetes mellitus (T2DM) (85) in overweight and obese individuals. Without question, the very high and further accelerating prevalence of obesity and obesity-related health complications carries an enormous economic burden. The direct costs of obesity on health care has reached nearly \$147 billion annually in the U.S. (84). Moreover, indirect costs associated with obesity (i.e. missed days of work, obesity related physician visits, etc.) are also high. For example absenteeism alone is believed to cost an additional \$4.3 billion annually. Unfortunately, the projected cost of obesity related diseases such as T2DM in the U.S. is also high (84). Estimates suggest that obesity-related chronic diseases will contribute to a rise in health care costs by a rate of \$22-68 billion per year through 2030 (94). Additionally, annual health care costs for individuals with diabetes is five times greater than that of individuals without diabetes and associated with \$174 billion annually in direct and indirect health care costs (1).

Although the increased disease risk associated with obesity is certainly caused by multiple factors, it appears that resistance to the physiologic actions of insulin (i.e., “insulin resistance”) underlies many obesity-related health complications including: T2DM, hypertension, dyslipidemia, as well as systemic and tissue inflammation (21, 27, 44, 64). Insulin resistance refers to the reduced ability of insulin to regulate nutrient metabolism in insulin-responsive tissues (e.g., skeletal muscle, liver, and adipose tissue). More specifically, insulin resistance in skeletal muscle results in an impaired

ability of insulin to promote glucose uptake; in the liver, insulin resistance induces glucose overproduction; while insulin resistance in adipose tissue suppresses the normally potent anti-lipolytic effects of insulin, resulting in an elevated lipolytic rate. Therefore, obesity-induced insulin resistance in these tissues results in hyperglycemia, hyperinsulinemia, and an elevated systemic fatty acid availability, which are well known to be particularly detrimental to vascular and metabolic health (21, 27). Hyperinsulinemia has also been found to increase blood pressure via vascular wall remodeling, vasoconstriction, and increased fluid retention (64). Insulin resistance is thought to be a major contributor to the development of dyslipidemia (27), and the onset of insulin resistance typically precedes dyslipidemia in obese individuals (27). Insulin resistant individuals have been reported to have 80% higher plasma triglyceride concentrations and 20% lower HDL-C concentrations than their insulin sensitive counterparts (44). Collectively these findings suggest that interventions aimed at improving insulin resistance could have important implications for improving overall health.

A physically active lifestyle can reduce disease risk

For at least the better part of the last century accumulating epidemiological evidence supports the notion that a more physically active lifestyle is linked with lower morbidity and mortality rates (11), some of which may be attributed to reduced prevalence of insulin resistance, T2DM (40) as well as other chronic diseases (95). The definition of a “physically active lifestyle” is very broad, and could range from regularly engaging in modest physical activity behaviors, like walking, to daily participation in very vigorous exercise training. Current exercise guidelines recommend at least 2.5 hours of moderate-to-vigorous physical activity each week to prevent T2DM (2). Unfortunately, those more vigorous forms of physical activity are associated with greater participant discomfort and drop-out rates (20). Light intensity physical activity however, appears to be the most prevalent form of physical activity (33, 50) and is also associated with significant reductions in risk for developing T2DM (39). In fact, compared with completely sedentary behavior, daily light physical activity such as walking is associated with a 34% reduction in the risk of developing T2DM (38) potentially resulting from better glucose tolerance and insulin sensitivity (34, 56). For example, a cross-sectional study observed a similar inverse relationship between light physical activity and plasma glucose concentrations following a 2hr oral glucose tolerance test (34). Further cross-sectional analyses have demonstrated similar increases in insulin

sensitivity in individuals who engage in more physical activity regardless of the intensity (56). Consequently, light intensity physical activity may be a very important therapeutic tool for improving glucose metabolism and ultimately reducing T2DM risk. A better understanding of the underlying mechanisms that contribute to the improvement in insulin resistance when people begin to incorporate light/mild intensity physical activity into their daily lives could have important health implications for designing lifestyle interventions aimed at reducing T2DM risk.

Current exercise guidelines emphasizing moderate-to-vigorous physical activity (2) in part stem from the understanding that regularly performed exercise at sufficiently high intensity can enhance cardiorespiratory fitness (e.g., maximal oxygen consumption (VO_{2max})), and higher cardiorespiratory fitness is associated with lower incidence of cardiovascular disease and T2DM even among overweight and obese individuals (51, 85). Therefore, the subsequent improvement in VO_{2max} resulting from regular physical activity/exercise is often credited for the exercise-induced improvements in health (51, 85). However, accumulating evidence suggests that many of the exercise-induced improvements in health may be independent of improved VO_{2max} (19, 23). For example, several weeks of moderate intensity endurance training has been found to improve measures of insulin resistance and dyslipidemia in overweight and obese individuals in the absence of improved VO_{2max} (23). Moreover, an overwhelming amount of evidence indicates that many of the effects of exercise on important health outcomes (e.g., insulin resistance, hypertension, dyslipidemia) are very transient (15, 35, 58). Many studies demonstrate that the beneficial health effects of exercise training dissipate after only a few days (or less) without exercise (35), well before meaningful decrements in VO_{2max} . Additionally, a single session of exercise can greatly improve insulin resistance and reduce blood pressure in overweight and obese individuals at least into the next day (19, 24). Yet one session of exercise is not enough of an exercise stimulus to enhance VO_{2max} within the same time period. Therefore, while it is clear that physical activity improves insulin resistance and metabolic health, the quantity or intensity of exercise required, and the underlying mechanisms responsible for this improvement are not completely understood. It remains uncertain how much of these health improvements are truly due to adaptations associated with the improvement in VO_{2max} , compared with some other attributes of physical activity, *per se*. Making this distinction becomes clinically relevant particularly when one considers that the vigorous activities/exercises required for robust improvements in VO_{2max} are

associated with participant discomfort and injury, which are thought to contribute to poor adherence (20).

Lipid oversupply and insulin resistance

The rate of fatty acid mobilization from adipose tissue (lipolysis) is markedly higher in obese compared with lean adults (37), and this over-abundance of fatty acids in the systemic circulation is an important link between obesity and the development of insulin resistance (79). Several studies have demonstrated that an elevation in plasma fatty acid availability via lipid and heparin infusion in lean, healthy adults results in profound insulin resistance within only 2-3 hours (22, 30, 72). Conversely, pharmacological suppression of lipolysis can improve insulin resistance in obese adults (69). The exact mechanisms by which elevated systemic fatty acid availability leads to skeletal muscle lipid accumulation and insulin resistance are not completely understood. Within skeletal muscle, in general fatty acids can either be oxidized in the mitochondria for energy production or stored in the form of intramyocellular triacylglycerols (IMTG) or lipid intermediates (i.e. diacylglycerol (DAG) and ceramide) (43). While the accumulation of neutral lipids within IMTG stores is now largely understood to be inert (61), the accumulation of DAG and ceramides have been more directly linked with impaired insulin signaling (Figure 1) (41, 80, 86, 88, 91). DAG and ceramide are bioactive lipid species capable of activating as well as inhibiting cellular signaling pathways (31). In the context of insulin resistance, DAG has been found to activate a novel protein kinase C (PKC) (41, 79), which has been implicated in serine phosphorylation of insulin receptor substrate-1 (IRS-1) (12) resulting in inhibition of the insulin signaling cascade, and the subsequent inhibition of translocation of the insulin-responsive glucose transporter (GLUT4) to the cell surface. The insulin desensitizing effects of ceramides appear to occur just downstream of IRS-1 by reducing the phosphorylation of protein kinase B (PKB; also known as Akt), and by altering the translocation of Akt to the plasma membrane through activation of novel PKCs (36). It has also been suggested that fatty acid-induced PKC activation may also increase other pro-inflammatory signaling pathways such as the Nuclear factor kappa-B (NF- κ B) pathway, thereby reducing insulin sensitivity (41). Notably, compared with non-obese individuals, obese individuals show greater basal DAG accumulation and activation of pro-inflammatory pathways (i.e. c-jun N-terminal kinase (JNK) and NF- κ B) (9, 59).

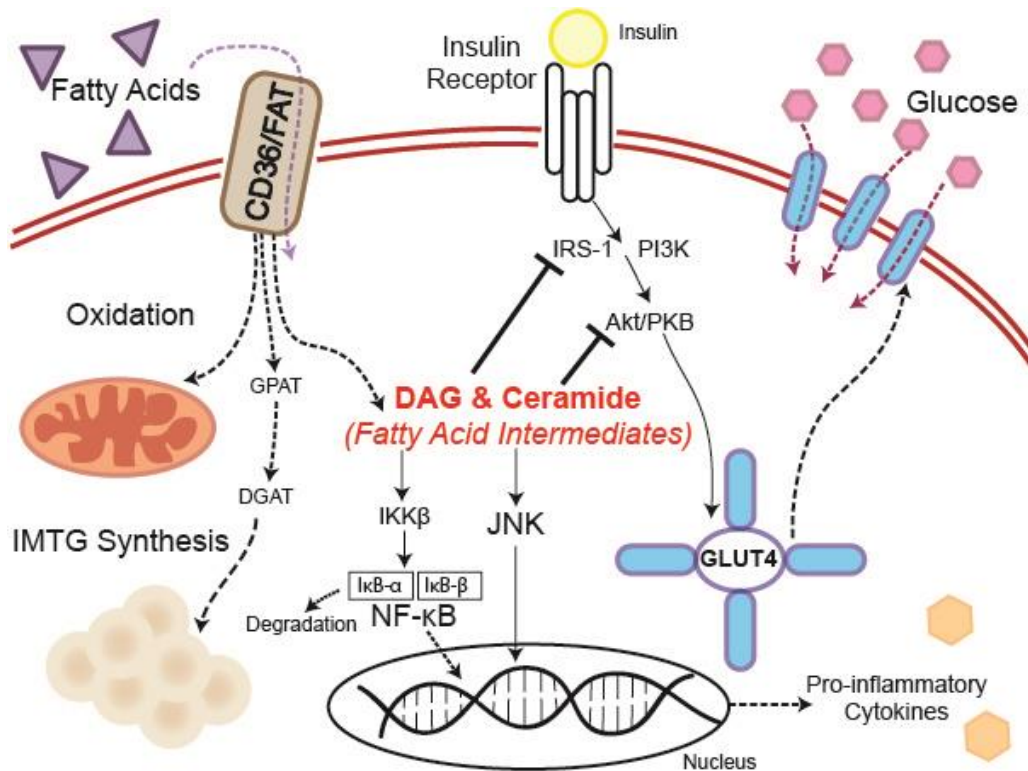


Figure 2-1. Influence of fatty acid intermediates (e.g., DAG, ceramide) on insulin signaling and glucose uptake.

Fatty acids enter the myocyte via facilitated diffusion, requiring transporter proteins such as CD36/FAT. In general, the two major fates of fatty acids within the myocyte are to either be oxidized within the mitochondria for energy or to be esterified and stored as IMTG. Fatty acids that are not oxidized or stored as neutral lipids can accumulate as bioactive lipid intermediates including DAG and ceramide. DAG can reduce insulin-stimulated glucose uptake by activating serine kinases (e.g., PKC), which serine phosphorylate IRS-1, thereby inhibiting insulin signaling and suppressing GLUT4 translocation to the cell membrane. Ceramides can impair insulin signaling by acting (directly and indirectly) on Akt/PKB; via either dephosphorylation of Akt and/or interfering with Akt translocation to the plasma membrane. DAG and ceramides can also activate IKK β , which phosphorylates I κ B α/β resulting in the dissociation of I κ B and NF κ B (and I κ B is subsequently degraded). Upon dissociation with I κ B, the liberated NF κ B is now able to translocate to the nucleus where it up-regulates the transcription of key pro-inflammatory cytokines (e.g., TNF α). DAG and ceramides can also activate JNK resulting in JNK's migration to the nucleus where it also promotes the production of pro-inflammatory cytokines. *Abbreviations:* GPAT, glycerol-3-phosphate acyltransferase; DGAT, diglyceride acyltransferase; IMTG, intramyocellular triacylglycerol; DAG, diacylglycerol; NF- κ B, nuclear factor kappa-B; I κ B, inhibitor of NF- κ B; IKK β , I κ B kinase; JNK, c-jun N-terminal kinase, PKC, protein kinase C; IRS-1, insulin receptor substrate-1; PI3K, Phosphatidylinositol 3-kinase; Akt/PKB, protein kinase B; GLUT4, glucose transporter type 4.

Exercise-mediated improvements in insulin resistance may be due in part to alterations in the metabolism of fatty acids within muscle, perhaps leading to a reduction in the accumulation of the “damaging” lipid intermediates. Our lab has demonstrated that an acute session of exercise protected against insulin resistance during a lipid infusion that was administered in the several hours after exercise (76). This protection against lipid-induced insulin resistance was accompanied by an increase in the accumulation of IMTG, and a reduction in DAG and ceramides, suggesting that the exercise session facilitated the partitioning of fatty acids that entered the muscle toward storage as neutral lipids in IMTG, rather than the other lipid intermediates. Our finding that the protein abundance of key enzymes in the IMTG synthesis pathway (i.e., glycerol-3-phosphate acyltransferase (GPAT) and diglyceride acyltransferase (DGAT)) were up regulated the morning after this single session of exercise helped to support this hypothesis (76). In conjunction with the reduced accumulation in muscle DAG and ceramide, we also found reduced phosphorylation/activation of JNK and increased abundance of IκB-α and IκB-β (76). Collectively these findings suggest that exercise may increase insulin sensitivity by acutely repartitioning fatty acids away from formation/accumulation of DAG and ceramides, and reduce markers of cellular inflammatory-stress.

It is important to note that the study described above (76) was performed in lean adults undergoing an overnight lipid/heparin infusion in attempt to mimic the elevated fatty acid availability in obesity. However, the responses to exercise in obese subjects, who are *chronically* exposed to elevated fatty acid availability, may be rather different. For example, we recently found that a single session of low intensity exercise (at 50% VO₂max) also improved insulin sensitivity the next day in obese individuals, but this improvement in insulin sensitivity was not accompanied by measurable reductions in DAG concentration (62). It is possible that the relatively large muscle lipid pools commonly found in obesity may make it very challenging to detect rather subtle (but perhaps physiologically meaningful) changes in muscle DAG abundance. But we also must acknowledge that the effects of exercise on lipid partitioning in obesity may differ from the effects we found in lean subjects who were acutely exposed to an overnight lipid infusion.

As described above, the insulin sensitizing effects of exercise training (and the subsequent improvement in aerobic capacity) are controversial, but much of the effects of exercise on insulin

sensitivity appear to stem from the most recent session(s) of exercise (58). In the context of exercise training and lipid-induced insulin resistance, there has been considerable debate about the role of increased oxidative capacity on the accumulation of intramyocellular lipids (66, 75, 77, 89). A greater accumulation of these lipids within muscle of obese individuals (5, 7) suggests a mismatch between fatty acid uptake and oxidation in skeletal muscle. Indeed, obese individuals are often found to have lower oxidative capacity than their non-obese counterparts (7, 47). Therefore, it has been proposed that the accumulation of lipid within skeletal muscle may be a consequence of lower oxidative capacity (10). However, increased oxidative capacity alone is not sufficient to improve skeletal muscle insulin resistance (75, 81). Therefore, although the contribution of a low oxidative capacity on muscle lipid accumulation and insulin resistance remains to be resolved, the negative impact of a high rate of fatty acid flux into the muscle is generally well accepted (6, 72, 79).

Influence of adipose tissue inflammation on insulin resistance

Obesity has been characterized as state of low-grade systemic inflammation that has been linked to the development of skeletal muscle insulin resistance (65, 78). Once thought of as simply a storage depot for excess nutrients, we now know that adipose tissue can also act as an important paracrine and endocrine organ, secreting peptides known to influence insulin resistance (42, 65, 78). Although the regulation and release of pro-inflammatory peptides (i.e. cytokines) from adipose tissue is not completely understood, much of the adipose-derived cytokine production in obesity may actually be produced in macrophages that infiltrate adipose tissue (42). In general there are two types of macrophages classically referred to as “M1” and “M2” macrophage, associated with pro- and anti-inflammatory protein secretion respectively. Compared with non-obese individuals, total macrophage abundance is higher in obesity (65) and there also appears to be a shift toward more M1 macrophage with obesity (52). Increased macrophage accumulation with adipose tissue may be at least partially explained by increased systemic availability of fatty acids that has been previously shown to increase adipose tissue macrophage accumulation (49). Additionally, hypertrophy of adipocytes, as seen in obesity, induces secretion of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF α), interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) (Figure 2-2) (42). Consequently, cytokines released within adipose tissue can influence inflammatory signaling in adipose tissue

and skeletal muscle through systemic circulation. Within adipose tissue, both TNF- α and IL-1 β have been found to promote lipolysis, resulting in liberation of both saturated and unsaturated fatty acids (28, 74). In turn saturated fatty acids have been shown to activate Toll Like Receptor-4 (TLR-4) resulting in the translocation of NF- κ B to the cell nucleus where it acts as a nuclear transcription factor in the production of more pro-inflammatory proteins (i.e., TNF- α , IL-1 β , IL-6, and MCP-1) from adipose tissue into systemic circulation. Furthermore, the cytokine MCP-1 specifically attracts more macrophage to adipose tissue (97). In addition, with increasing adipocyte cell volume with weight gain and obesity there is a reduction in adipose tissue blood flow (8) which can induce hypoxia (97). Hypoxia can activate both transcription factors HIF1- α and NF- κ B, which have been linked with increasing M1 macrophage infiltration and pro-inflammatory cytokine production respectively (Figure 2-3).

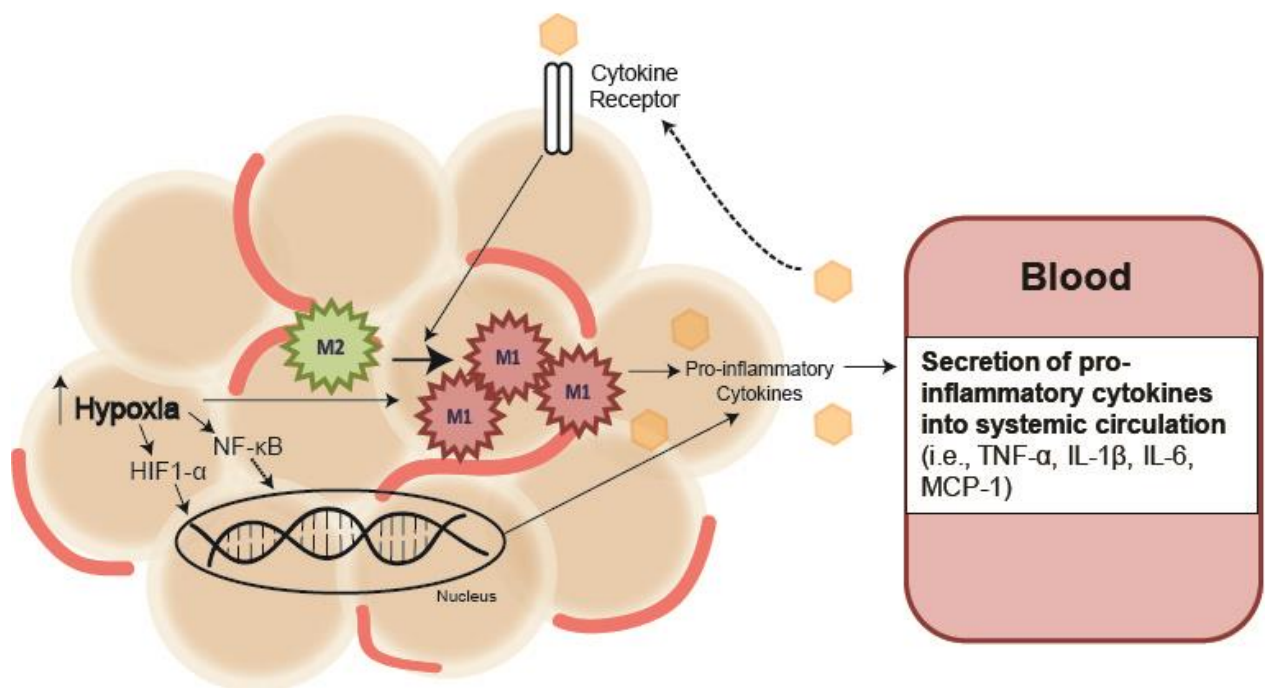


Figure 2-2. Adipose tissue inflammatory pathways leading to increased systemic circulation of pro-inflammatory cytokines. As adipocytes hypertrophy with weight gain and obesity, M1 macrophages are preferentially recruited to adipose tissue, and the ratio of M1-to-M2 macrophage increases. M1 macrophages produce pro-inflammatory cytokines, while M2 macrophage are considered anti-inflammatory. In addition, with increasing adipocyte cell volume, capillary density diminishes, which can induce hypoxia. Hypoxia activates transcription factor HIF1- α , which has been linked with increasing M1 macrophage infiltration and augmenting inflammation in adipose tissue. Hypoxia can also induce translocation of NF- κ B and the secretion of pro-inflammatory cytokines as previously described. *Abbreviations:* HIF1- α , hypoxia-inducible factor-1, alpha; M1, pro-inflammatory macrophage; M2, anti-inflammatory macrophage; TNF- α , tumor necrosis factor-1 alpha; IL-1 β , interleukin-1 beta; IL-6, interleukin-6; MCP-1, monocyte monocyte chemoattractant protein-1.

Systemic pro-inflammatory cytokines (i.e. TNF- α , IL-6, and MCP-1) are positively associated with measures of insulin resistance (3, 46, 53), but how these cytokines influence insulin resistance within skeletal muscle is not completely understood. Similar to the inflammatory response within adipose tissue, available evidence suggests that pro-inflammatory cytokines can directly influence skeletal muscle insulin resistance through inhibition of the insulin signaling cascade or indirectly through activation of pro-inflammatory pathways that may circulate back and inhibit insulin signaling (Figure 3) (96). In fact, it has been found that macrophage accumulation is also elevated in skeletal muscle of obese individuals, and their abundance is inversely associated with insulin sensitivity (92). It is also known that TLR-4 expression is elevated in obese insulin resistance individuals (71). In vitro studies using human derived myocytes have shown that physiological concentrations of IL-1 β and IL-6 are sufficient to inhibit insulin signaling at the level of Akt/PKB serine phosphorylation and MCP-1 can reduce insulin stimulated glucose uptake (82). Importantly many pro-inflammatory cytokines (i.e., TNF- α , IL-1 β , IL-6, and MCP-1) are known to be produced endogenously within skeletal muscle (13). This makes it difficult to determine the influence of adipose tissue and skeletal muscle derived pro-inflammatory cytokines on insulin signaling. This is especially so because in vivo models of inflammation and insulin signaling or glucose uptake have focused primarily on either adipose tissue or skeletal muscle (45, 53, 71, 92). Therefore, assessing both adipose tissue and skeletal muscle inflammation in obese individuals may contribute to our understanding in the relationship between inflammation and skeletal muscle insulin resistance.

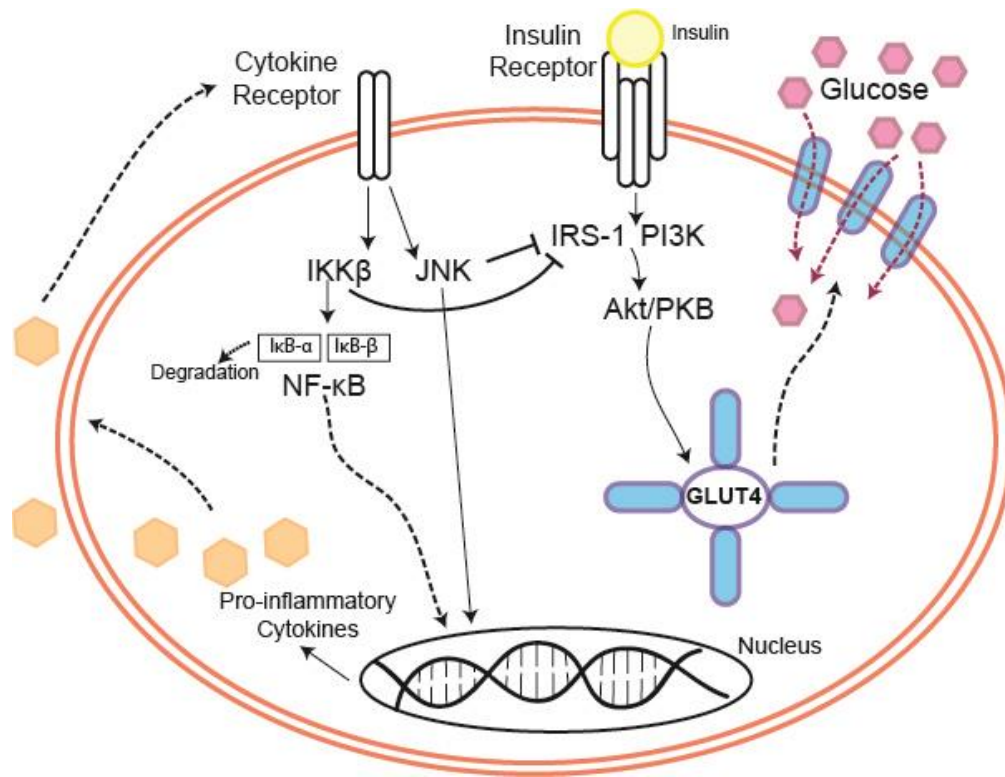


Figure 2-3. Influence of pro-inflammatory cytokines on skeletal muscle insulin resistance.

Pro-inflammatory cytokines (e.g., $\text{TNF}\alpha$, $\text{IL-1}\beta$) can inhibit insulin stimulated glucose uptake within skeletal muscle through activation of $\text{IKK}\beta$ and JNK resulting in serine phosphorylation of IRS-1 , thereby inhibiting insulin signaling and suppress GLUT4 translocation to the cell membrane. These cytokines can also work more indirectly by facilitating the ability of $\text{NF-}\kappa\text{B}$ and JNK to migrate the nucleus, which can increase the transcription of additional inflammatory cytokines. These cytokines may then exit the cell and interact with cell surface membrane receptors (in an autocrine and paracrine manner), further perpetuating the pro-inflammatory cycle and the inhibition of the insulin signaling. Abbreviations: $\text{NF-}\kappa\text{B}$, nuclear factor kappa-B; $\text{I}\kappa\text{B}$, inhibitor of $\text{NF-}\kappa\text{B}$; $\text{IKK}\beta$, $\text{I}\kappa\text{B}$ kinase; JNK , c-jun N-terminal kinase, PKC , protein kinase C; IRS-1 , insulin receptor substrate-1; PI3K , Phosphatidylinositol 3-kinase; Akt/PKB , protein kinase B; GLUT4 , glucose transporter type 4.

Influence of acute and habitual physical activity on adipose tissue, systemic, and skeletal muscle inflammation

The effects of exercise/physical activity on the abundance of pro-inflammatory cytokines in adipose tissue, systemic circulation, and skeletal muscle of obese individuals remain unresolved. In adipose tissue, hypoxia has been proposed to be a major mediator of inflammation through activation of the NF κ B pathway (97) and by reducing efflux of macrophage from adipocytes (90). A potent stimulator of angiogenesis, exercise may reduce pro-inflammatory signaling in adipose tissue through enhanced blood flow. Hypoxia inducible-factor 1 (HIF-1) is a transcription factor and master regulator of genes involved in oxygen homeostasis inducing expression of hypoxia inducible-factor 1 α (HIF-1 α) leading to the transcription of vascular endothelial growth factor (VEGF) and ultimately increased angiogenesis (83). Obese individuals show reduced adipose tissue blood flow (57) and increased expression of HIF-1 α (14). At least in rodent models exercise training does appear to increase capillary density of adipose tissue (32) and reduce M1 macrophage as well as TLR-4 expression (45). However, to our knowledge markers of hypoxia and vascularization have not been examined in response to exercise training in obese individuals. If exercise training can influence the inflammatory profile within adipose tissue it does not appear to modulate the systemic pro-inflammatory state of obese individuals who demonstrate no change in IL-6, TNF- α , or MCP-1 with 12 weeks of exercise training when measured 24-48 hrs after participants last exercise training session (16). A better understanding of the inflammatory response to acute and habitual physical activity within adipose tissue and in the systemic circulation of obese individuals could have important implications for designing lifestyle interventions aimed at reducing disease risk associated with chronic inflammation.

Much less is known about the inflammatory state of muscle in response to physical activity/exercise training in overweight and obese humans. Macrophage accumulation is higher in skeletal muscle of obese individuals (92) and capable of endogenous production of pro-inflammatory cytokines (96). Acutely, exercise stimulates the production and secretion of IL-6 from skeletal muscle (25). However, the release of these cytokines peaks at the end of exercise and is relatively low in the basal state of lean individuals (25). It is not clear whether macrophages are the source of IL-6 production in skeletal muscle. Monocytes are precursor cells for macrophage and also classically characterized as pro- and anti-inflammatory (68). Overweight and obese individuals show greater markers of pro-inflammatory monocytes (87). While exercise

training has been shown to reduce the percentage and concentration of pro-inflammatory monocytes (87), expression of macrophage and pro-inflammatory cytokines within skeletal muscle remains unchanged (16). Therefore, it is not clear how acute and habitual physical activity influence markers of inflammation in skeletal muscle. Determining the inflammatory state of adipose tissue, systemic circulation, as well as skeletal muscle could provide a more complete understanding of how exercise/physical activity contributes to improved cardiovascular and metabolic health (Figure 2-4).

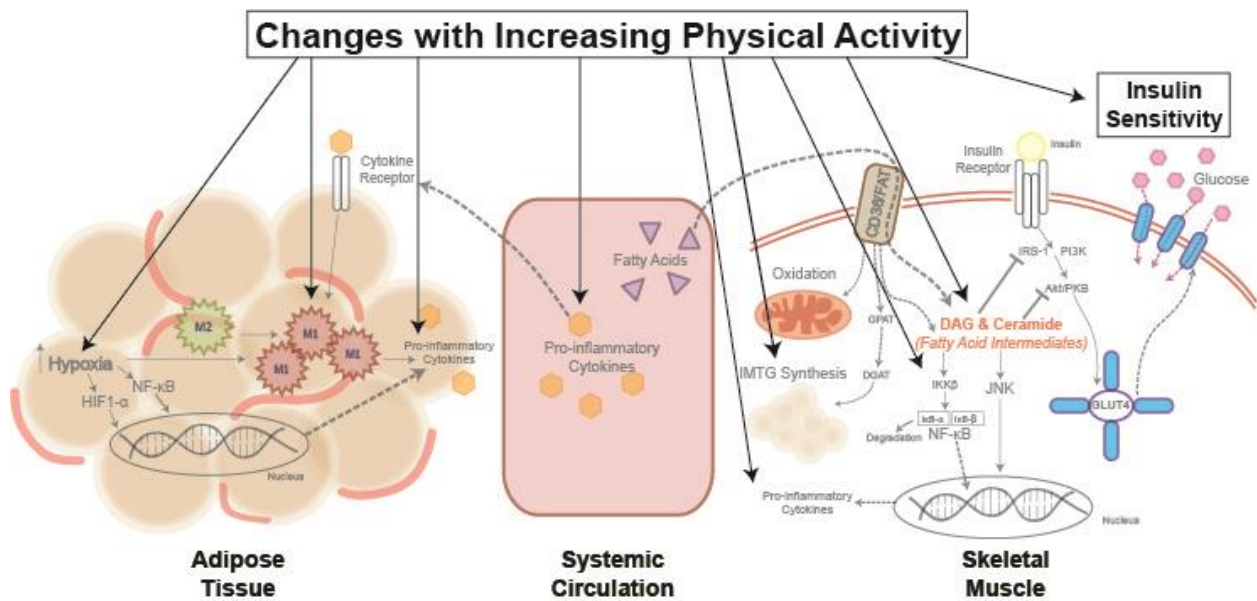


Figure 2-4. Potential targets for assessing adipose tissue, systemic and skeletal muscle inflammation in response to acute exercise and habitual physical activity. My dissertation projects were designed to examine the effects of acute exercise and habitual physical activity on insulin sensitivity, and markers for inflammatory pathway activation that may affect insulin action in muscle. In addition, I also explored the effects of acute exercise and habitual physical activity (without weight loss) on pro-inflammatory cytokine profile within adipose tissue and systemic circulation of overweight and obese individuals.

Measure of insulin resistance – strengths and limitations

Insulin resistance and insulin sensitivity are major outcomes for the projects of my dissertation. Many different methods have been used, and validated, to assess insulin resistance in humans. These methods range from rather crude assessments based on fasting plasma glucose and insulin concentrations e.g., Homeostatic Model Assessment of Insulin Resistance [HOMA-IR], to more involved/sophisticated methods requiring glucose and insulin infusion (e.g., hyperinsulinemic-euglycemic clamp). Although HOMA-IR has been reported to correspond well with insulin resistance measured using the “gold-standard” clamp procedure (93), there are very important limitations of using HOMA-IR in part because this measure only involves fasting glucose and insulin rather than changes in glucose metabolism in response to an increase in insulin. However, HOMA-IR has been acknowledged to be a useful tool for assessing insulin sensitivity in large-scale epidemiological studies (54, 93) such as the NHANES databases used in PROJECT #1 of my dissertation.

A more involved method of estimating insulin resistance includes measurements of plasma glucose and insulin concentration during an oral glucose tolerance test (OGTT). Calculations estimating whole-body, muscle and hepatic insulin sensitivity have been derived from the OGTT and compare reasonably well with measures acquired with the hyperinsulinemic-euglycemic clamp (4, 55). The product of glucose and insulin area under the curve (AUC) within the first 30 minutes of the OGTT has been used as a measure of hepatic insulin resistance (4). In healthy individuals the rise in plasma glucose and insulin within the first 30 minutes of the OGTT should be sufficient to suppress endogenous glucose production from the liver. Therefore, elevated plasma and insulin concentrations within that time frame reflect the inability of insulin to suppress endogenous production of glucose from the liver.

$$\text{Hepatic Insulin Sensitivity Index} = [\text{Glucose AUC}_{0-30}] \times [\text{Insulin AUC}_{0-30}]$$

As noted, this method of estimating hepatic insulin resistance has been shown to be significantly correlated with more direct measures of hepatic insulin resistance (4). Likewise, assuming hepatic glucose production is nearly completely suppressed 30 minutes into the OGTT, the reduction in plasma glucose concentration would reflect peripheral glucose uptake. Therefore, the decline in

glucose concentration from peak to nadir during the OGTT, divided by the average insulin concentration during this timeframe has been used as an indicator of peripheral insulin resistance and is also significantly associated with more sensitive measures of peripheral insulin sensitivity (4).

$$\text{Muscle Insulin Sensitivity Index} = \frac{dG}{dt} / [\text{MPI}]$$

In addition to these tissue-specific indices of insulin sensitivity, the Matsuda Composite Index of Insulin Sensitivity (ISI) provides a measure of whole body insulin sensitivity as a product of fasting plasma glucose and insulin concentration and average plasma glucose and insulin concentration in response to a glucose load (55). This calculation is derived from the assumption that fasting glucose concentration is indicative of endogenous glucose production from the liver as a function of insulin concentration and the average plasma glucose and insulin concentration during the OGTT reflects both the suppression of hepatic glucose production and disposal of glucose in peripheral tissues.

Matsuda Composite Index

$$= 10,000 / \sqrt{[(\text{fasting insulin} \times \text{fasting glucose}) \times (2\text{h glucose} \times 2\text{h insulin})]}$$

Similarly, ISI is also significantly correlated to the hyperinsulinemic-euglycemic clamp method of measuring insulin resistance (55). Unlike both HOMA-IR and the hyperinsulinemic-euglycemic clamp method of determining insulin resistance, ISI is a dynamic measure of insulin resistance in response to a glucose load potentially reflecting a normal meal for an individual. Therefore, the glucose and insulin response to a glucose load similar to a “meal” may have very important clinical implications in populations at increased risk for T2DM. Also the OGTT has been repeatedly found to respond fairly quickly (within a day) to activity and inactivity (35, 73) suggesting that it would be an appropriate measure of insulin resistance for PROJECT #2 of my dissertation examining the acute and chronic effects of exercise on insulin resistance in overweight and obese adults.

The most sensitive measure of insulin resistance, and currently the “gold standard” for determining insulin resistance, is the hyperinsulinemic-euglycemic clamp method (60). The hyperinsulinemic-euglycemic clamp method involves two intravenous catheters in contra-lateral arms for a constant infusion of insulin and variable infusion of glucose as well as frequent arterialized blood draws at a rate of one every five minutes (18, 60). Additionally a stable isotope tracer infusion of glucose can also be used to distinguish between hepatic and peripheral insulin resistance (60). Because of the level of involvement of the clamp procedure, it is not ideal for large epidemiological or clinical investigations (60). However, there are two important advantages to the hyperinsulinemic-euglycemic clamp method including its relatively high degree of reproducibility and low variability (60). This makes the hyperinsulinemic-euglycemic clamp method ideal for smaller experimental investigations examining subtle yet potentially meaningful changes in insulin resistance similar to the study proposed in PROJECT #3 of my dissertation examining the progressive adaptations associated with adapting a fairly modest exercise training program in obese adults.

Summary of review of literature

The overall objective of my dissertation projects was to thoroughly examine key metabolic adaptations that are consistent with a habitually active lifestyle. These findings will have important implications for designing lifestyle interventions specifically aimed at enhancing metabolic health in overweight individuals. Although increased physical activity is clearly known to improve metabolic health, the optimal amount of exercise required, and the underlying mechanisms contributing to this improvement are not completely understood. Most current exercise recommendations are designed with the goal to improve cardiorespiratory fitness (e.g. maximal oxygen uptake (VO_{2max})) (2). However, mounting evidence suggests many health benefits of exercise are largely independent of improvements in VO_{2max} , suggesting a training program designed to improve “fitness” may not be optimal for improving metabolic health. This is particularly relevant because the higher intensity exercise required to improve VO_{2max} often leads to poor exercise adherence (20). Additionally, many of the metabolic health benefits of exercise actually stem from the most recent exercise session(s), rather than from adaptations to weeks, months, and even years of exercise training (19, 24). Insulin resistance is also linked to the development of many other chronic diseases, and it is a primary outcome measure of my

dissertation studies (21, 27, 44, 63). Despite clear evidence that insulin resistance is greatly improved after a vigorous session of exercise (i.e., 24-48 hrs after exercise – but mostly dissipates after that), the effects of cumulative sessions of a more modest exercise/physical activity program on metabolic responses and adaptations that may improve insulin resistance remain unclear.

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CHAPTER 3

Daily physical activity predicts degree of insulin resistance: a cross-sectional observational study using the 2003–2004 National Health and Nutrition Examination Survey

Abstract

This study examined the independent association of objectively measured physical activity on insulin resistance while controlling for confounding variables including: cardiorespiratory fitness, adiposity, sex, age, and smoking status. Data were obtained from National Health and Nutrition Examination Survey 2003-2004, a cross-sectional observational study conducted by the National Center for Health Statistics of the Centers for Disease Control that uses a stratified, multistage probability design to obtain a nationally representative sample of the U.S. population. The analysis included 402 healthy U.S. adults with valid accelerometer, cardiorespiratory fitness, and fasting plasma glucose and insulin concentrations. After controlling for relevant confounding variables we performed a multiple linear regression to predict homeostatic model of insulin resistance (HOMA-IR) based on average daily minutes of moderate-to-vigorous physical activity (MVPA). In our bivariate models, MVPA, cardiorespiratory fitness and body fat percentage were all significantly correlated with log HOMA-IR. In the complete model including MVPA and relevant confounding variables, there were strong and significant associations between MVPA and log HOMA-IR ($\beta = -0.1607$, $P = 0.004$). In contrast the association between cardiorespiratory fitness and log HOMA-IR was not significant. In conclusion, when using an objective measure of physical activity the amount of time engaged in daily physical activity was associated with lower insulin resistance, whereas higher cardiorespiratory fitness was not. These results suggest that the amount of time engaged in moderate-to-vigorous physical activity may be an important determinant for improving glucose metabolism.

Keywords: Ambulatory monitoring, physical fitness, adiposity

Introduction

The incidence of obesity-related diseases, such as type 2 diabetes is increasing in parallel with the alarming rise in the prevalence of obesity (16). Lifestyle programs involving weight loss and exercise are often found to improve insulin resistance (IR) in individuals with diabetes, and also to prevent or delay the onset of diabetes in those at risk of developing the disease (2). Although weight loss can markedly improve IR, exercise can also improve IR even in the absence of weight loss. Additionally, although improved cardiovascular “fitness” in overweight and obese individuals is linked with a reduced incidence of diabetes, exercise can also improve IR without enhancing cardiovascular fitness (11). For example, a single session of exercise can have a profound improvement on IR that can persist for several hours and even days (7, 25). Collectively this evidence suggests that exercise, per se, provides a potent stimulus for improving IR. Therefore, promoting a lifestyle change to increase regular physical activity (PA), even if it is not sufficient to induce weight loss or improve cardiovascular fitness, may be a viable and realistic intervention option aimed at reducing diabetes risk.

“PA” is broadly defined to include any bodily movement produced by skeletal muscle that increases energy expenditure (1). Until recently it has been difficult to quantify ambulatory PA in free-living individuals. Large cohort studies have traditionally used self-reported surveys to measure PA, contributing to errors in determining the intensity and duration of PA (12, 23). However, objectively measured PA has been shown to be a better predictor of metabolic health than self-reported PA (5). Accelerometers, currently the gold standard for objectively measuring PA in a free-living population (26), were incorporated into the National Health and Nutrition Examination Survey (NHANES) 2003-2004 survey, a nationally representative sample of residence in the U.S. Analysis of fasting blood samples (including plasma concentrations of insulin and glucose), and other variables known to influence IR (i.e. cardiorespiratory fitness, and total body adiposity) were also determined for a subset of healthy participants in this NHANES cohort. The objective measurement of these constructs in a large representative cohort of U.S. adults allows more precise estimates than in previous studies along with an opportunity to access lower intensity PA that is difficult to quantify by self-report. Therefore, the aim of our study was to examine the independent association of objectively measured PA on IR while controlling for potential confounding variables in a representative sample of healthy U.S. adults.

Methods

Inclusion criteria

NHANES is a cross-sectional observational study conducted by the National Center for Health Statistics of the Centers for Disease Control and Prevention that uses a stratified, multistage probability design to obtain a nationally representative sample of the U.S. population. The survey population included randomly selected households within clusters of neighborhoods. Of the 12,761 individuals selected during the 2003-2004 survey, 10,122 individuals agreed to participate. The NHANES 2003-2004 survey included an interview, physical examination and laboratory testing conducted by trained staff. Only non-pregnant adults aged 18-49 without a history of diabetes (or taking medication to treat diabetes), cardiovascular or renal disease, stroke or emphysema were included in this analysis. Because participants were scheduled for either a morning blood draw after a 9-hour fast or an afternoon blood draw after a 6-hour fast, a fasting questionnaire was used to determine whether or not participants met the fasting requirements. Therefore, fasting glucose and insulin used to calculate the primary outcome of homeostatic model of IR (HOMA-IR) were only available on approximately half of the NHANES participants who were classified as fasted. Also, only participants who successfully completed submaximal VO₂max fitness test in order to determine cardiorespiratory fitness were included in this analysis (Figure 3-1).

Assessment of insulin resistance

Blood samples were obtained by trained medical personnel in mobile examination centers after a 6- or 9-hour fast. Samples were centrifuged; the plasma from each sample was placed into storage test tubes, shipped to the University of Missouri-Columbia (Columbia, Missouri), and stored at -70° until analysis. Plasma glucose concentration was determined by hexokinase enzyme method. Plasma insulin concentrations were measured with Tosoh AIA-PACK IRI (Toyama, Japan) two-site immunoenzymometric assay. Individuals with fasting insulin concentrations < 2 µIU/mL or > 100 µIU/mL (n = 34) were considered outliers and were excluded from the analysis. HOMA-IR (the product of fasting plasma glucose and insulin concentrations divided by 22.5 [mM*µIU/mL/22.5]) was used as a composite index for IR (34).

Assessment of habitual physical activity

PA was monitored by an Actigraph AM-7164 accelerometer (formerly the CSA/MTI AM-7164, manufactured by ActiGraph of Ft. Walton Beach, FL), which is a pager-sized device powered by a small lithium battery and attached to an elasticized belt worn on the right hip (33). The accelerometer measured the duration and intensity of PA by capturing the magnitude of acceleration (intensity) and summing the magnitudes (intensity counts) within a specified time interval (epoch). A one-minute epoch was used by NHANES (17). Participants were asked to wear the device for seven consecutive days while they were awake and to remove it while swimming or bathing. Monitors were returned by express mail to NHANES, where data were downloaded and the device was checked to determine whether it was still within manufacturer's calibration specifications. NHANES used standardized data quality procedures to assess validity and reliability of Actigraph ACC data (17). Our analysis included PA data from participants who wore the accelerometer for at least 600 minutes on four or more days of the week. Any block of time greater than or equal to 60 minutes where the activity count was equal to zero was considered time when the monitor was not worn. Each minute of accelerometer data was coded based on the recorded activity counts for that minute. Minutes with ≥ 1952 activity counts were coded as moderate-to-vigorous intensity PA (MVPA) and ≥ 260 and < 1952 activity counts were coded as light PA (24). We summed the number of minutes at these intensities over the entire day. This activity did not have to represent clearly defined sessions of exercise or be sequential and were thus accumulated throughout the day. Minutes of activity were divided by 30 to reduce the risk of rounding error in regression betas due to the fine scale of activity minutes. This calculation yields a measure of PA in which each unit represents approximately 30 minutes of PA per day. Changes to the units do not affect the associations described by our analysis.

Assessment of cardiorespiratory fitness

A multiple-stage submaximal treadmill exercise test was employed to estimate maximal oxygen consumption (VO₂max), which was used as the marker for cardiorespiratory fitness. Per NHANES protocol, inclusion criteria for exercise testing were based on medical conditions, medication and physical limitations determined during household interviews, and limited to adults 18-49 years who did not have any known cardiac conditions. A detailed list of inclusion criteria and submaximal exercise testing procedures can be found elsewhere (4). Briefly, the exercise test

included a two-minute warm-up on the treadmill, two separate three-minute submaximal exercise stages and a two-minute cool-down. Heart rate associated with known workloads at the end of each stage were used to estimate VO₂max, as previously described (4). Grade and speed for each stage were selected based on age, sex, body mass, and self reported PA. Heart rate was monitored throughout the test by four electrodes attached to the participant's thorax and abdomen. Individuals with non-physiological estimates of VO₂max (> 100 ml/kg/min, n=3) were excluded from the analysis.

Anthropometric measures

A wall-mounted stadiometer and a digital floor scale were used to measure height and weight, respectively, and calibrated as previously described (31). Body fat percentage was determined by dual-energy X-ray absorptiometry (DEXA) using Hologic QDR-4500A fan-beam densitometer (Hologic, Inc., Bedford, Massachusetts) (3). The DEXA was performed by a certified radiology technologist, and the densitometer was calibrated daily with a Hologic Anthropomorphic Spine Phantom as directed by the manufacturer.

Smoking status

Smoking status was determined by measuring plasma serum levels of cotinine, which is a major metabolite of nicotine. Individuals with cotinine levels > 10 ng/ml were coded as being current smokers.

Participant characteristics

Age at the time of the survey was calculated from the participant's self-reported date of birth. Participants were classified into 1 of 4 categories for race/ethnicity based on self-reported background: Non-Hispanic white, Non-Hispanic black, Mexican American, or other.

Statistical analysis

Descriptive statistics include means, standard deviations, and range for continuous variables (glucose, insulin, HOMA-IR, PA, VO₂max, percent body fat, and age). Frequencies were calculated for categorical variables (i.e. sex and race/ethnicity). Because HOMA-IR was not normally distributed, we used log HOMA-IR as the primary outcome variable in our regressions.

Multiple linear regressions were performed with log HOMA-IR as the dependent variable. HOMA-IR was transformed using the log function to correct for the skewed distribution. Minutes of MVPA and VO₂max were the primary predictor variables. Appropriate confounding variables including: adiposity, sex, and age were also included in our complete model. Model parameters were also stratified by sex. Race/ethnicity and smoking status did not significantly impact model estimates and was not included in any reported models. Adjusted r-squared values were calculated during the addition of confounding variables to our complete model. We also examined variance inflation factors to determine multicollinearity in our complete model.

Predicted mean values of HOMA-IR with confidence intervals were calculated for various levels of PA using the complete multiple linear regression including PA, cardiorespiratory fitness, percent body fat, age, and sex as independent variables.

All analysis took into consideration NHANES complex survey design including: weighting, stratification, and clustering. Sample weights for the NHANES 2003-2004 fasting sample were used. For all analysis, significance was set at <0.05. Statistical analysis was performed using Stata 11.0 for Windows (StataCorp LP, College Station, TX, 2006).

Results

Participant characteristics

Of the 10,122 participants in the 2003-2004 NHANES sample, 883 were healthy, non-pregnant adults between the ages of 18-49 who were scheduled for a morning fasting blood draw. Of those 883 participants, 402 had valid data for HOMA-IR calculations, estimated VO₂max, and PA (Figure 3-1). Categorical and continuous summary statistics of the final sample are presented in Table 3-1. The 481 individuals who were excluded from the analysis were slightly older, heavier and more likely to be non-white than the 402 with complete data (Table 3-1).

Regression models

Parameter estimates for the four different regression models were presented in Table 3-2. We did observe a weak but significant relationship between cardiorespiratory fitness and physical activity ($r^2=0.1065$, $P<0.01$; Figure 3-2). Importantly variables in our complete model did not demonstrate

multicollinearity with an average variance inflation factor of 1.55 (range: 1.05-2.19). In bivariate models, MVPA and cardiorespiratory fitness were all significantly correlated with log HOMA-IR with higher levels of MVPA ($\beta=-0.224$, $P=0.024$) and cardiorespiratory fitness ($\beta=-0.019$, $P=0.003$) predicting lower log HOMA-IRs. However, in our complete model (Model 4), including predicting and confounding variables (cardiovascular fitness, body fat percentage, age and sex), the association between cardiorespiratory fitness and log HOMA-IR became non-significant while MVPA remained significantly correlated with log HOMA-IR. When stratified by sex, our analysis did not show any evidence of moderation by sex on the association between MVPA and log HOMA-IR (data not shown).

Light physical activity

We also examined minutes of light PA in our analysis. Although not statistically significant, there was a trend for an association between LPA and log HOMA-IR in our bivariate model ($\beta=0.0288$, $P=0.099$) and for LPA to predict log HOMA-IR when included in a more complete model with confounding variables ($\beta=-0.0214$, $P=0.093$). Importantly, when both LPA & MVPA were modeled with confounding variables, MVPA remained a significant predictor of log HOMA-IR ($\beta=0.1457$, $P=0.035$) while LPA ($\beta=-0.0154$, $P=0.184$) and cardiovascular fitness ($\beta=0.0015$, $P=0.760$) did not.

Prediction of HOMA-IR based on Model 4.

The predicted HOMA-IR means with confidence intervals for a range of MVPA levels are presented in Table 3-3. Assuming that the association found in Model 4 is causal, if the average individual in our model (based on means presented in Table 1 for predicting and confounding variables) who initially does not engage in any MVPA and subsequently increases his/her PA to 30 minutes of MVPA a day can decrease their HOMA-IR by 0.26 which is an 13% reduction in HOMA-IR.

Additional correlational analyses

Please note that in the process of responding to reviewers' comments for this submitted manuscript (which is now accepted for publication in the *International Journal of Behavioral Nutrition and Physical Activity*), it was requested that we remove some comparisons from the accepted

manuscript that I had included in my original dissertation proposal. These additional comparisons were to examine the independent association between physical activity and mean arterial pressure (MAP), total cholesterol (Total-C), and C-reactive protein as well as the independent association between cardiorespiratory fitness and these same outcomes. These data can be found in APPENDIX 1.

Discussion

Although increased PA is known to improve IR, the mechanisms underlying this improvement are not completely understood. More specifically, it is difficult to distinguish the metabolic benefits of PA, per se, from the improvement in cardiorespiratory fitness that often accompanies an increase in PA. Presently, we found a strong and significant association between daily MVPA and a measure of IR (HOMA-IR) after adjusting for confounding variables including cardiorespiratory fitness. In contrast, we did not observe the same relationship between cardiorespiratory fitness and log HOMA-IR after correcting for the same confounding variables. Together these results highlight the potential importance of daily MVPA as a target intervention of improving IR independent of changes to cardiorespiratory fitness.

It is clear from previously published longitudinal studies that individuals with greater cardiorespiratory fitness are at lower risk for developing diabetes (30, 32). Yet improvements in cardiovascular fitness as a result of exercise training are highly variable and can take several months to develop (6). Also, while the most robust improvements in cardiovascular fitness stem from higher intensity training (8, 10), this kind of activity is also associated with greater participant discomfort and injury contributing to higher dropout rates (8). Therefore, it is necessary to determine alternative intervention programs that may be more attainable by individuals at risk of developing diabetes. Our prediction analysis shows marked improvements in IR with relatively modest changes to daily MVPA. Indeed the benefits of cardiorespiratory fitness on other health outcomes such as cardiovascular health are undeniable (12, 23), but modest PA may be an appropriate alternative for individuals that may see exercise intensity as a barrier to being more physically active.

Near-optimal fasting plasma glucose and insulin levels yield a HOMA-IR value close to 1, and our model showed that 120 minutes of MVPA per day predicts near optimal HOMA-IR values. This is equivalent to only 7.5 minutes of activity per hour during wakeful hours (accounting for 8 hours of sleep), throughout the day. Some PA guidelines emphasize a bout of at least ten minutes of PA to improve health (18). However, our analysis included accumulated minutes of PA throughout the entire day. We did not determine how the MVPA was accumulated, and future research is required to determine whether MVPA accumulated sporadically throughout the day is sufficient to improve IR or if it should be accumulated in bouts to have a positive effect on IR.

Surprisingly, LPA was not significantly associated with IR in our regression models. In contrast, similar methods, using objectively measured PA, identical criteria for defining intensity of PA, and inclusion of confounding variables like adiposity (i.e. waist circumference or BMI), have reported an inverse relationship between LPA and 2-hour plasma glucose concentrations during an oral glucose tolerance test (19). This discrepancy between our results and previous analysis may be explained by the use of HOMA-IR, which has not been found to be associated with LPA (20). Furthermore, while the use of accelerometers are currently the ideal method for measuring PA and have a high degree of sensitivity for LPA, HOMA-IR is a less sensitive measure of IR. Therefore LPA could have a positive effect on IR that we were unable to detect in our analysis. The use of more sensitive measures of IR (i.e. intravenous glucose tolerance test and hyperinsulinemic-euglycemic clamp methods) may be required to determine the influence of LPA on IR. Further investigation into the influence of LPA on IR may have important public health implications for individuals incapable or resistant to higher intensity PA.

Obesity is associated with increased risk of IR and type 2 diabetes (21, 29). In our complete model, we show that body fat percentage was most strongly associated with IR. With two-thirds of the U.S. population classified as overweight or obese, weight loss is an important therapeutic target for metabolic disease prevention (15, 16). In fact modest weight loss (~5% of initial body weight) can induce clinically relevant improvements in metabolic health (22). However, clinical trials targeting behavioral changes to diet and exercise as well as pharmacological interventions have shown only modest weight loss and more importantly have proven difficult to sustain (9). Because

sustained weight loss is so difficult for many people (9), adopting a physically active lifestyle may be a more feasible alternative to weight loss for those at increased risk of developing IR.

Our study has certain limitations. Because our analysis is cross sectional, causation cannot be determined. However, our analysis showed that higher MVPA was indicative of lower IR in healthy adults. Large observational trials confirm the association between PA and HOMA-IR (13). Controlled intervention trials in which adiposity, cardiorespiratory fitness and PA are manipulated independently in a sample large enough to convincingly establish causal association are likely to be infeasible. However, a more practical approach may be to systematically determine the minimum “dose” (i.e. intensity, duration, caloric expenditure, etc.) of PA, as well as the effect of habitual PA over time on the mechanisms underlying an improvement in IR.

It is also important to note that HOMA-IR is not the most sensitive measure of insulin resistance, and better measures of insulin resistance exist including an intravenous glucose tolerance test and hyperinsulinemic-euglycemic clamp methods. Although our analysis included a relatively insensitive measure of insulin resistance, we were able to detect measurable differences and the HOMA-IR method was practical for measuring insulin resistance in a population this large and diverse.

The NHANES 2003-2004 survey limited cardiovascular fitness testing to individuals <49 yrs of age because of increased risk for an adverse cardiovascular incident. Examining the independent relationship between physical activity and cardiovascular fitness on insulin resistance in middle and older aged adults is an important question worth pursuing. However, we were unable to make this analysis with the current NHANES data set.

Because only healthy adults were selected for this analysis in order to rule out any unnecessary confounding factors brought about by disease, on average our sample was more active and fit than the average American. While participants averaged nearly 30 minutes of MVPA per day and had an estimated VO_{2max} of $40 \pm 9 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, there was a large range of values and high variability between participants, suggesting a rather heterogeneous subject pool rather than a highly fit population. Although a maximal exercise test was not performed, VO_{2max} was estimated

objectively using a submaximal exercise test. Submaximal exercise tests have been shown to be strongly correlated with maximal measures of VO_2max (14). Also, although the participants in the present study were generally younger than the age range where the incidence of newly diagnosed type 2 diabetes is greatest (45-64 years old (1)), in order to prevent or delay the onset of diabetes it is important to determine how PA behavior may influence IR in younger adults (>45 year old). Other than requiring an overnight fast, diet and exercise were also not controlled prior to participants' fasting blood draw or submaximal exercise testing. Although macronutrient meal composition and exercise can acutely alter IR (25, 28) it is not unreasonable to assume that participant's diets and exercise habits were not altered during this brief testing period.

Our analysis, using objective measures of PA demonstrated that an increase in daily MVPA had a significant and positive effect on IR. Importantly these improvements were independent of other variables also known to influence diabetes risk including cardiovascular fitness and adiposity. Therefore, if improved glucose metabolism is the primary goal, lifestyle programs targeting improvements in metabolic health may be best designed to encourage individuals to participate in daily MVPA. This may be particularly important for individuals who have difficulty achieving or maintaining weight loss and/or for those who may be deterred by vigorous exercise regimens.

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| Variable | Final Cohort Mean \pm Linearized SE (n=402) | Range (n=402) | Excluded Individuals Mean \pm Linearized SE (n=481) |
|---|---|------------------|---|
| Sex (%) | | | |
| Male | 53.0 | | 49.0 |
| Female | 47.0 | | 51.0 |
| Race (%) | | | |
| Non-Hispanic white | 74.0 | | 68.0* |
| Non-Hispanic black | 10.0 | | 14.0* |
| Mexican American | 10.0 | | 8.0* |
| Other | 7.0 | | 10.0* |
| Smoking Status | | | |
| Smokers | 29.0 | | 37.0 |
| Non-smokers | 71.0 | | 63.0 |
| Age (yrs.) | 32.6 \pm 0.56 | 18-49 | 34.0 \pm 0.51* |
| Mass (kg) | 79.7 \pm 1.16 | 42.8-156.7 | 82.8 \pm 0.95 |
| BMI ^a (kg/m ²) | 26.9 \pm 0.26 | 16.0-50.1 | 28.3 \pm 0.29* |
| Body Fat (%) | 31.2 \pm 0.40 | 12.2-51.8 | 33.2 \pm 0.42* |
| Fasting glucose (mmol/L) | 5.1 \pm 0.03 | 3.8-12.1 | |
| Fasting insulin (μ U/mL) | 9.4 \pm 0.49 | 2.0-61.2 | |
| HOMA-IR ^b | 2.1 \pm 0.11 | 0.2-17.3 | |
| Average MVPA ^c (min) | 32.1 \pm 1.23 | 0.0-134.0 | |
| Estimated VO ₂ max ^d (ml/kg/min) | 39.7 \pm 0.54 | 21.4-78.9 | |

Table 3-1. Participant demographics, anthropometric, metabolic, physical activity characteristics and cardiorespiratory fitness (n=402)

^a body mass index

^b homeostatic model assessment of insulin resistance

^c moderate-to-vigorous physical activity

^d maximal oxygen consumption

*Statistically significant difference between final cohort (n=402) with valid measures of HOMA-IR, cardiorespiratory fitness, and physical activity and those excluded due to missing or invalid data.

| Beta (P-value) | Model 1 | Model 2 | Model 3 | Model 4 |
|--|----------------------------------|----------------------------------|-------------------------------------|--------------------------------------|
| Physical Activity (30 min of MVPA ^b) | -0.2241 (0.024) | -0.1676 (0.072) | -0.0680 (0.378) | -0.1607 (0.028) |
| Estimated VO ₂ max ^c (ml/kg/min) | | -0.0144 (0.018) | 0.0035 (0.613) | -0.0008 (0.868) |
| Body Fat (%) | | | 0.0396 (<0.001) | 0.0789 (<0.001) |
| Age (yrs.) | | | | -0.01611 (0.005) |
| Sex Male = 1; Female = 0 | | | | -1.0540 (<0.001) |
| Adjusted r ² | 0.042 | 0.064 | 0.165 | 0.423 |
| r ² (% change) | | 52.4% | 158% | 156% |

Table 3-2. Linear regression models predicting log of insulin resistance (log HOMA-IR^a). For all models n=402.

^a homeostatic model assessment of insulin resistance

^b moderate-to-vigorous physical activity

^c maximal oxygen consumption

| Average MVPA (min/day) | HOMA-IR | 95% CI |
|-----------------------------------|----------------|---------------|
| 0 | 1.95 | 1.58-2.32 |
| 15 | 1.81 | 1.57-2.06 |
| 30 | 1.69 | 1.52-1.86 |
| 45 | 1.57 | 1.40-1.73 |
| 60 | 1.46 | 1.25-1.69 |
| 75 | 1.35 | 1.08-1.63 |
| 90 | 1.26 | 0.93-1.59 |
| 105 | 1.17 | 0.79-1.55 |
| 120 | 1.09 | 0.67-1.51 |

Table 3-3. Predicted effect of average minutes of MVPA^a on HOMA-IR^b and 95% confidence interval (95% CI)

Predictions are based on Model 4

^a moderate-to-vigorous physical activity

^b homeostatic model assessment of insulin resistance

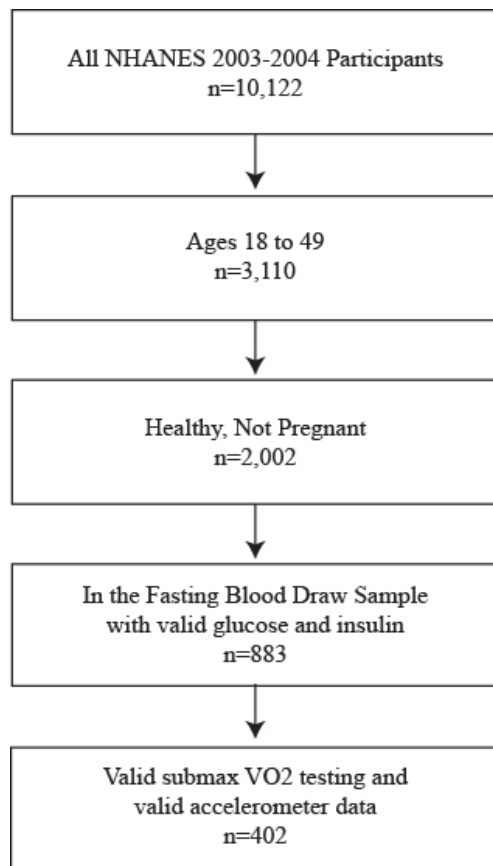


Figure 3-1. Inclusion (subsample) flow chart.

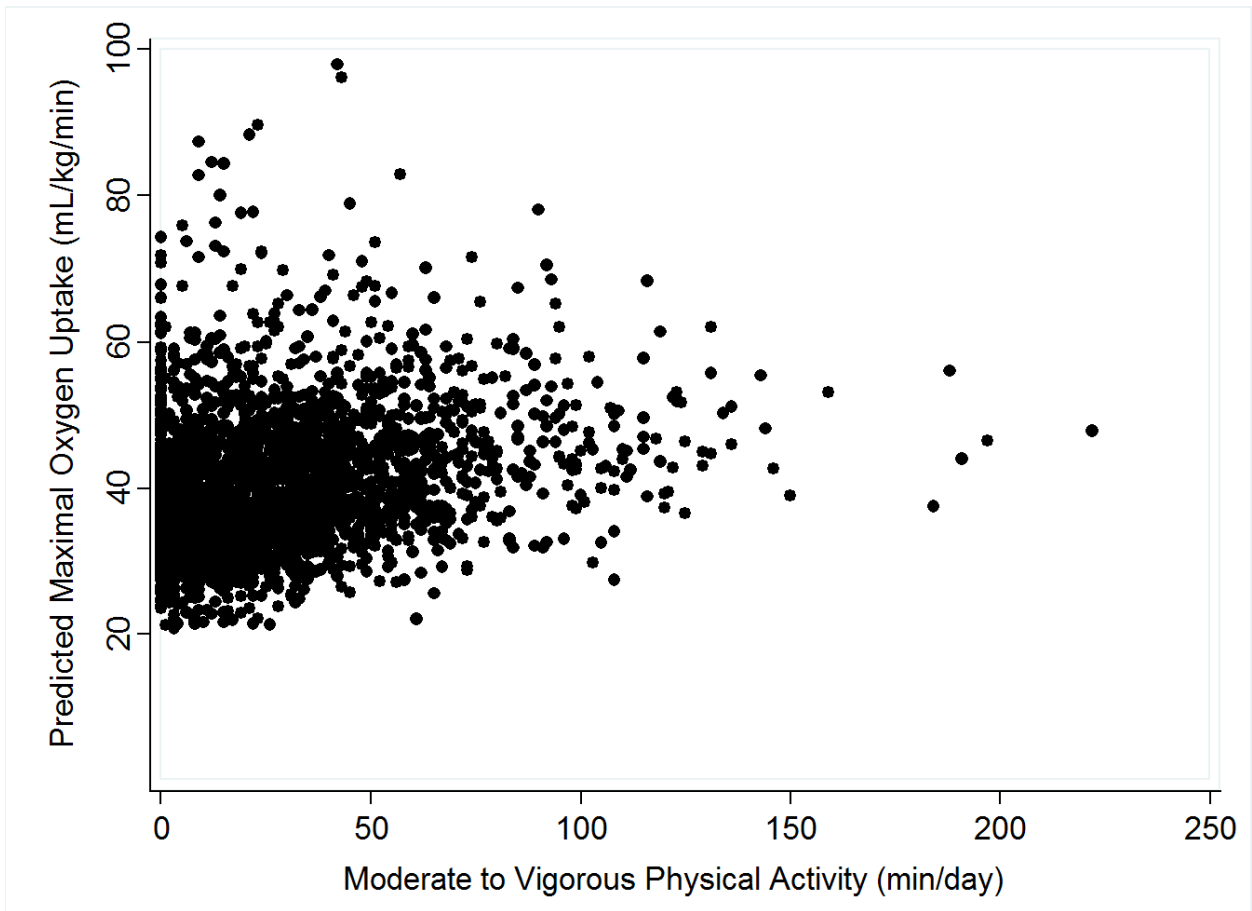


Figure 3-2. Relationship between average minutes of daily MVPA and cardiorespiratory fitness.

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CHAPTER 4

Health benefits of habitual and acute exercise in overweight-to-mildly-obese adults

Abstract

The aims of this study were to compare markers of cardiometabolic disease risk in overweight adults who exercise regularly with a well-matched cohort of overweight adults who do not exercise, and to assess the metabolic impact of a single session of exercise in these groups. Twenty-eight overweight-to-mildly obese men and women (BMI: 27-34 kg/m²) were divided into 2 cohorts based on their self-reported exercise behavior. 16 of these subjects (male/female: 7/9) were classified as exercisers (“EX”) (>2.5h planned endurance-type exercise/week), while 12 subjects (male/female: 4/8) were non-exercisers (“Non-EX”). VO₂max was significantly greater in EX vs. Non-EX (33±3 vs. 25±2 ml/kg/min, p=0.04) but as designed, body mass (89±3 vs. 88±3 kg), %body fat (34±1 vs. 37±2%), and waist circumference (100±2 vs. 96±2 kg) were well-matched between groups. Participants reported to the laboratory after an overnight fast, and we measured resting blood pressure (BP), a fasting blood sample was collected for measures of blood lipids and inflammatory cytokines, and insulin sensitivity was assessed using the Matsuda Insulin Sensitivity Index (ISI) during an oral glucose tolerance test (OGTT). Importantly, EX participants refrained from exercising for exactly 3 days before the measurements. After the OGTT, all participants performed a single session of exercise (1h at ~70% of their age-predicted HR_{max}). They returned to the laboratory the next morning for the same measurements performed the day before. ISI was significantly higher in EX vs. Non-EX (3.3±0.3 vs. 2.5±0.4; P=0.03), but BP, and blood lipids were not different between groups. The acute exercise session increased ISI the next morning in Non-EX (2.5±0.4 vs. 3.2±0.6; P=0.01), and this improvement in ISI was significantly correlated with the reduction in the plasma concentration of the pro-inflammatory cytokine, IL-1β (P=0.05). The exercise session did not increase ISI the next day in EX (3.1±0.2 vs. 3.3±0.5; P=0.56), and as a result, ISI was similar between groups the morning after a single session of exercise. In summary, exercising regularly (>2.5h/week) was associated with a persistent improvement in insulin sensitivity among overweight adults, but not blood pressure or blood lipids. Additionally, just one

session of exercise increased insulin sensitivity among sedentary overweight adults to levels equal to regular exercisers.

Keywords: *Exercise training, physical activity, sedentary, insulin resistance*

Introduction

Two-thirds of the US adult population are now classified as overweight or obese (33), and unfortunately, obesity rates are continuing to rise (34). Along with the alarming rise in obesity rates is the incidence of obesity related cardiometabolic complications including insulin resistance, dyslipidemia, hypertension as well as chronic systemic inflammation (85, 97). Although modest weight loss (5-10% of initial body weight) has been found to reduce cardiometabolic disease risk (59), even this very modest weight loss can be very difficult to both achieve and sustain (23). Alternatively, physical activity/exercise has been highlighted as an important therapeutic tool for reducing cardiometabolic disease risk among overweight and obese adults (1). While regular exercise has been identified as a very important factor in delaying and/or preventing cardiometabolic disease risk, it is surprising that the factors underlying the health improvements associated with regular exercise are still not well understood, particularly in overweight and obese adults.

Although a physically active lifestyle is commonly associated with a lower incidence of cardiometabolic diseases (45-47, 64), it is often the exercise-induced improvement in VO₂max (i.e., maximal oxygen consumption) that is credited for the reduced disease risk. However, endurance training has been found to improve measures of insulin resistance, dyslipidemia, and blood pressure in overweight and obese individuals in the absence of improved VO₂max (26, 38, 92). Additionally, in PROJECT #1 we found that average minutes of daily moderate-to-vigorous physical activity, rather than VO₂max, was significantly associated with improved insulin resistance, suggesting that regular physical activity rather than improved VO₂max helps mediate the improvement in cardiometabolic health. Moreover, the effects of exercise on cardiometabolic risk factors (e.g. insulin resistance, hypertension) may be very transient, dissipating after only a few days (or less) without exercise (40, 57, 71, 72, 104). Additionally, evidence also exists to suggest that a single session of exercise may improve insulin resistance, as well as reduce blood lipids and blood pressure in people who do not exercise regularly (16, 22, 71, 75, 100). Therefore,

although regular exercise is clearly an important contributor to long-term health and disease prevention, it remains unclear how much of these health benefits stem from the acute effects of the most recent session(s) of exercise, and how much can be attributed to physiologic adaptations accrued as a result of habitual exercise.

Obesity is now often characterized as a chronic state of low-grade inflammation, which is linked to increased cardiometabolic disease risk, including insulin resistance (8, 90, 102). Adipose tissue is a main source of pro-inflammatory cytokine production in overweight and obese adults. Although the regulation and release of pro-inflammatory cytokines from adipose tissue is not completely understood, much of the adipose-derived cytokine production in obesity may actually be produced in macrophages that infiltrate adipose tissue (49). Regardless of the source (i.e., adipocytes vs. macrophage) obese adults are often found to have elevated plasma concentrations of many pro-inflammatory cytokines (e.g., interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF- α), and interleukin-1 beta (IL-1 β)) (55, 73, 80, 88). As described above, exercise can reduce cardiometabolic disease risk but the influence of a regular exercise routine and acute exercise on markers of systemic inflammation within overweight adults is unclear. A better understanding of the inflammatory response to habitual and acute exercise in overweight and obese adults could have important implications for designing lifestyle interventions aimed at reducing cardiometabolic disease risk.

The overall aims of this study were to compare markers of cardiometabolic disease risk in overweight adults who exercise regularly with a well-matched cohort of overweight adults who do not exercise, and to assess the metabolic impact of a single session of exercise in these groups. In general, we hypothesized that the protective effects of regular exercise on cardiometabolic disease risk factors would stem largely from the most recent session(s) of exercise. Along these lines, we hypothesized that when overweight adults who exercise regularly were required to abstain from exercise for three days, insulin sensitivity, blood pressure, fasting blood lipids, and markers of systemic inflammation would be similar when compared with a well-matched cohort of habitually sedentary overweight adults. Additionally, we hypothesized that a single session of exercise would induce similar improvements in cardiometabolic risk factors measured the next day in both the regular exercisers and the habitual non-exercisers.

Methods

Subjects A total of 28 overweight-to-obese (body mass index [BMI]: 27-34 kg/m²) men (n=11) and women (n=17) participated in this study. Subjects were divided into 2 cohorts based on their self-reported habitual exercise. Sixteen subjects (male/female: 4/8) were classified as “Exercisers” (EX; >2.5 h of planned endurance-type exercise/week; male/female: 7/9), while 12 subjects were “Non-exercisers” (Non-EX; no planned exercise). Inclusion criteria included no history of metabolic or cardiovascular disease and weight stable (i.e., ± 2 kg for ≥ 6 months). Participants were not taking any medications (with the exception of oral contraceptives). Female participants were scheduled during the follicular phase of their menstrual cycle for the experimental trial. Written informed consent was obtained from all subjects prior to participation. All procedures were approved by the University of Michigan Institutional Review Board.

Preliminary testing

Participants completed a physical activity questionnaire (see APPENDIX 2) in order to classify them as either EX (≥ 2.5 h of moderate-to-vigorous exercise/week for >6 months) or Non-EX (no planned exercise). Importantly, individuals who reported performing planned exercise between ≥ 30 min/wk but < 2.5 h/wk were excluded from the study. Within one month of the experimental protocol, participants completed a sub-maximal exercise test to predict VO₂max (as a marker of cardiorespiratory fitness) (30). We also measured each subject’s body composition using hydrostatic weighing technique (17).

All subjects participated in a two-day experimental trial performed on consecutive days (Figure 4-1). In order to assess the persistent effects of a regular exercise on our major outcome measures, subjects in the EX group abstained from their normal exercise routine exactly 3 days before Day 1 of the experimental trial, and then abstained from their exercise program until after the experiment was complete. The day before the experimental trial (Day 0), all participants wore a pedometer (New Lifestyles, Lee’s Summit, MO) and were instructed to remain relatively sedentary by completing < 5000 steps per day (94). Participants also wore an ambulatory blood pressure monitor (A&D Company, Tokyo, Japan) – to assess blood pressure every 30 minutes between 2pm-10pm during the afternoon/evening before the experiment. A standardized meal (30% of total daily energy requirements; ~ 750 kcal) eaten at 1800h and a standardized snack ($\sim 10\%$ of

daily energy requirements) eaten at 2100h were provided to participants and they consumed only water for the remainder of Day 0.

Subjects returned to the laboratory at 0700h the next morning after an overnight fast (Day 1). After resting quietly for 30 minutes, a resting blood pressure measurement was obtained (A&D Company, Tokyo, Japan). We then placed an intravenous (IV) catheter in an arm vein and collected a baseline blood sample to determine plasma substrate and hormone concentrations (i.e. glucose and insulin), blood lipid profile (i.e., plasma concentrations of HDL-C, LDL-C, total cholesterol, triglycerides, fatty acids), and plasma markers of systemic inflammation (i.e., TNF- α , IL-1 β , IL-6, and MCP-1). After the baseline blood sample was withdrawn we performed a two-hour oral glucose tolerance test (OGTT) with a 75g glucose load (Glucose Tolerance Test Beverage, Fisher Scientific). Blood samples were collected every 15 minutes for the duration of the test. After the OGTT, the IV was removed and participants were provided a standardized breakfast (see “Study diets” section below) and rested quietly. At noon, participants performed a session of exercise on a treadmill for 1 hour at a moderate intensity equal to 70% of their age-predicted heart rate max (HRmax). After completing the exercise session, subjects were provided a standardized lunch, eaten in the laboratory. They were also provided another standardized meal and a snack to be eaten at home at 1800h and 2100h, respectively. Identical to the afternoon of Day 0, participants again wore a pedometer and ambulatory blood pressure monitor. Subjects returned to the laboratory at 0700h the next morning (Day 2) after an overnight fast – at which time we measured their resting blood pressure, collected a baseline blood sample and performed another 2 h OGTT to determine the responses the day after a single session of exercise in both cohorts.

Study diets

Importantly, diets were designed to maintain "energy balance" (i.e.; energy intake = energy expenditure) to avoid the confounding influence of a negative energy balance on insulin sensitivity (5). In the evening of Day 0 (i.e., the evening before the experiment), the total energy content of the standardized dinner and snack were ~30% and ~10% of estimated daily energy expenditure, which was assessed using calculations from Cunningham, et al., (20). Because the exercise session on Day 1 increased daily energy expenditure, we calculated Day 1 energy expenditure for each

participant as follows: $[(VO_2 \text{ during exercise}) \times (5\text{kcal/liter of } O_2 \text{ consumed}) \times 60\text{min} + [(1.5 \times (370 + (21.6 \times \text{FFM})))] \times (1380\text{min})]$, and energy intake on Day 1 was provided to match this calculated energy expenditure. On Day 1, breakfast (including the OGTT), lunch, and dinner each contained ~30% of participants estimated daily energy expenditure. The evening snack contained ~10% of participants estimated daily energy expenditure. After the evening snack on Day 0 and Day 1, subjects did not eat anything until completion of the OGTT the next day. The macronutrient composition of meals and snacks was: 55% carbohydrate, 30% fat, and 15% protein, which represents the macronutrient content of a “typical” western diet (9).

Analytical procedures

Plasma substrate and hormone concentrations

Blood samples were collected in chilled EDTA tubes, centrifuged (1,600 g for 20 min. at 4°C) within 30 min of collection, and then stored at -80°C until analysis. Plasma glucose (glucose oxidase assay; Thermo Fisher Scientific, Waltham, MO), fatty acid (NEFA-HR assay kit; WAKO Life Sciences, Inc., Richmond, VA), triglyceride (Triglyceride Reagent; Sigma Adrich, St. Louis, MO), total- and high-density lipoprotein (HDL; Cholesterol E and HDL-Cholesterol E; WAKO Life Sciences, Inc.) concentration were measured with commercially available colorimetric assay kits. Plasma insulin concentration was measured using a commercially available radioimmunoassay (RIA) kit (Human insulin RIA kit; EMD Millipore, Billerica, MA). Plasma markers of systemic inflammation (e.g. TNF- α , IL-1 β , IL-6, and MCP-1) were measured using commercially available Multiplex magnetic bead kits (EMD Millipore) and the Luminex L200 instrument (Luminex, Austin, TX). Data from the Multiplex bead kits were quantified using xPONENT software (Luminex). All assay kits were used per manufacturer instructions.

Calculations

Index of whole body insulin sensitivity: Plasma glucose and insulin concentrations measured immediately before and during the OGTT were used to assess insulin sensitivity using the Matsuda Composite Index (69). The Matsuda Composite Index has been found to be a reasonably accurate marker for insulin sensitivity when compared with the hyperinsulinemic-euglycemic clamp (69).

Matsuda Composite Index

$$= 10000/\sqrt{[(\text{fasting insulin} \times \text{fasting glucose}) \times (\text{2h glucose} \times \text{2h insulin})]}$$

We also calculated whole-body insulin resistance using the Homeostatic Model Assessment of Insulin resistance (HOMA-IR), which provided a supplementary index of insulin action in our subjects (105).

$$\text{HOMA} - \text{IR} = (\text{fasting plasma glucose} \times \text{fasting plasma insulin})/22.5)$$

Low density lipoprotein cholesterol concentration (LDL-C): Plasma LDL-C was calculated from measurements of plasma concentrations of total cholesterol (Total-C), HDL-C, and triglyceride

$$[\text{LDL-C}] = ([\text{Total-C}] - [\text{HDL-C}] - [\text{Triglyceride}])/5$$

Percent body fat: Body volume and density were assessed by hydrostatic weighing technique and the Siri equation was used to determine body fat percentage (17).

Statistical analysis

Student's t-tests were used to compare baseline characteristics between participants in our EX and Non-EX cohorts. A two-way ANOVA with one factor repeating (group x day) with Tukey post-hoc analysis was used to assess significant differences in insulin sensitivity, blood pressure, markers of systemic inflammation, and plasma lipid concentrations between cohorts and in response to the single session of exercise. ISI was not normally distributed and was therefore log transformed prior to statistical analysis (untransformed data are presented). A P-value of ≤ 0.05 was considered statistically significant.

Results

Subject characteristics and intensity during the experimental exercise session

As designed, subjects in our EX and Non-EX cohorts were very well matched for body weight, BMI, and body composition (Table 4-1), but there was a great disparity in the planned exercise between the groups. While our Non-EX subjects reported no planned exercise our EX group averaged >32 MET-hours per week of planned exercise (Table 1). In conjunction with their greater habitual exercise, VO₂max in EX was 36% greater than Non-EX (P<0.05; Table 4-1). The exercise session on Day 1 of the study was performed at the same relative intensity in the EX and Non-EX subjects (i.e., same %HRmax and same %VO₂max; Table 4-2). However, because of the difference in VO₂max between the groups, the absolute exercise intensity in EX tended to be greater than Non-EX, as noted by a trend for a higher absolute VO₂ during exercise (Table 4-2) and total energy expended during exercise (468 ± 42 vs. 384 ± 35 kcals for EX and Non-EX, respectively), but differences between groups did not reach statistical significance (P=0.15).

Insulin Sensitivity Index

Insulin sensitivity in our subjects appeared to be influenced by their habitual physical activity behavior. Despite similar body weight and adiposity, Insulin Sensitivity Index (ISI) was significantly higher in EX than Non-EX (Figure 4-2). Importantly, the EX participants abstained from exercise for 3 days in order to washout the effects of their most recent session of exercise. The calculation of HOMA-IR supported the notion that insulin sensitivity was higher in our EX vs Non-EX subjects (HOMA-IR: 3.4 ± 0.3 vs. 4.5 ± 0.5, respectively; P=0.05). Importantly, the single session of exercise performed in the afternoon of Day 1 of the experiment increased ISI the next morning in Non-EX, but not EX (Figure 4-2). As a result of this increase in ISI only in the Non-EX group, there was no longer a difference in ISI between EX and Non-EX the day after the single session of exercise (Day 2).

Markers of Systemic Inflammation

In the morning of Day 1 of the experiment, fasting plasma concentrations of IL-6, MCP-1, and TNF-α were not different between Non-EX and EX (Figure 4-3 A-C). In contrast, IL-1β tended to be higher in Non-EX compared with EX on Day 1, but this did not quite reach statistical significance (2.9±0.7 vs. 5.2±1.3, P=0.09, Figure 4-3 D). Interestingly, the improvement in ISI

found the morning after acute exercise in our Non-Ex group was significantly correlated with the change in plasma IL-1 β concentration (P=0.05; Figure 4-4). There was no such relationship between the change in ISI and any of the other inflammatory markers measured for either the EX or Non-EX subjects.

Blood Pressure and blood lipid profile

Habitual physical activity did not appear to affect blood pressure in our subjects because both resting and ambulatory blood pressures were very similar between EX and Non-EX on Day 1 (Figure 4-5). The single session of exercise reduced resting diastolic blood pressure the next morning, but this reduction only reached statistical significance in the EX cohort (Figure 4-5 B). We also found a trend for ambulatory systolic blood pressure to be reduced following a single exercise session in the Non-EX group but this did not reach statistical significance (P=0.10; Figure 4-5 C). Although regular exercise is often associated with reduced cardiovascular disease risk (28), we did not detect differences in blood lipids (i.e., Total-C, HDL-C, LDL-C, triglyceride) between our EX and Non-EX groups, and the acute exercise session also did not significantly alter the plasma concentration of these blood lipids in either group (Table 4-3). We did observe a very slight, yet significant reduction in fasting plasma NEFA concentration the morning after a single session of exercise in our EX cohort (Table 4-3).

Discussion

A physically active lifestyle is widely identified as being an important contributor to improved health and disease prevention (47, 106). However, it is still unclear whether these health benefits stem largely from acute responses to the most recent session(s) of exercise or from more persistent effects due to physiological adaptations that accrue in response to regular exercise. Our findings indicate that in contrast to our hypothesis, overweight adults who exercise regularly were more insulin sensitive than their well-matched sedentary counterparts, even when the acute effects of exercise were removed by abstaining from exercise for 3 days. Therefore, unlike findings from studies indicating that the insulin sensitizing effects of exercise training wear off after even a few days without exercise (40, 57, 71, 72, 81, 103), we found a persistent effect of regularly performed exercise on insulin action in our weight-stable overweight adults. Interestingly, we also found that a single session of relatively moderate-intensity exercise did not further enhance the insulin

sensitivity index (ISI) the next morning in these subjects who were already regular exercisers. In contrast, the single exercise session did increase ISI the next morning in our habitually sedentary subjects, and this improvement in ISI was associated with a reduction in the systemic pro-inflammatory factor IL-1 β . This suggests that the insulin sensitizing effect of acute exercise in overweight adults who do not regularly engage in exercise, may be mediated in part by a reduction in their systemic inflammatory state.

Many studies report that the increase in insulin sensitivity with exercise training is very transient, often returning to levels found in non-exercisers after only three days without exercise (40, 57, 71, 72, 81, 103). In contrast, we found that ISI was elevated in our group of exercisers compared with their well-matched cohort of non-exercisers, even when they did not exercise for a few days. It is conceivable that some of this discrepancy between our findings and these previous studies may be explained by the possibility that some effects of acute exercise may still persist 3 days after the exercise in our habitually active subjects. However, Oshida al., (81) reported that the acute effects of exercise on insulin action were gone 38h after the most recent session of exercise. Perhaps more importantly, these earlier studies reporting short-lived improvements after exercise were largely performed in lean subjects (40, 57, 71, 72, 81, 103), and the response in overweight adults may be different. In lean humans and animals, the exercise-induced reduction in muscle glycogen is associated with the enhanced insulin action found after exercise (12, 31, 84). For example, in lean animals the time course of muscle glycogen resynthesis after exercise tracks very closely with the time course of the transient exercise-mediated increase in insulin sensitivity (14). Alternatively, because insulin action is often found to be impaired in overweight/obese adults (10, 11) it seems plausible that habitual exercise in overweight adults may induce some longer lasting adaptations that may counteract their insulin resistance. Several studies suggest that an exercise-induced increase in oxidative capacity may contribute to an improvement in insulin action by increasing oxidative disposal of fatty acids – thereby reducing the accumulation lipid intermediates known to impair insulin signaling (56, 82, 98, 99). But the impact of an increase in oxidative capacity on resting fatty acid metabolism and insulin sensitivity is debated (35, 43, 44, 101). Alternatively, a previous study from our lab (89) reported that exercise altered the metabolic fate of the fatty acids within skeletal muscle by enhancing storage as neutral lipid rather than accumulation of the other lipid intermediates known to impair insulin signaling (42). Alterations in muscle fatty acid

metabolism may be especially relevant in overweight/obese adults, and may extend (and perhaps prolong) their exercise-induced improvement in insulin sensitivity beyond the effects of lower muscle glycogen concentration. However, the improvement in insulin resistance with habitual exercise in overweight/obese adults may not be isolated to adaptations occurring exclusively in the exercising muscles. For example, hepatic insulin resistance has been found to improve with exercise training (58). Additionally, exercise training in obese adults has been found to increase adiponectin (54), an adipose tissue-derived cytokine known to enhance whole body insulin sensitivity (66). Therefore, regularly performed exercise in overweight/obese adults induces adaptations in multiple tissues that may contribute to a more persistent improvement in insulin sensitivity, even after the exercise-induced reduction in muscle glycogen concentration has been replenished.

Consistent with previous reports (22, 71, 75) we found that a single session of exercise was sufficient to improve insulin sensitivity the next day in overweight adults who do not exercise regularly. In contrast, we did not observe an improvement in ISI after exercise in regular exercisers, and as a result, the difference in ISI between groups was no longer apparent the day after exercise. The mechanisms underlying the improvement in ISI in response to chronic vs. acute exercise are likely very different (described in more detail below). However, the clinical relevance of this finding is intriguing because it suggests that when compared with the enhanced insulin action stemming from exercising routinely, a similar degree of improved insulin action can be achieved immediately, without having to wait for the accrual of adaptations in response to months or years of exercise training. Over time, as an individual adopts a more active lifestyle, the mechanisms underlying the exercise-induced improvement in insulin action may change, but the clinical outcome remains the same (i.e., improved insulin sensitivity). However, it is important to note that the ISI measured in our group of regularly exercising overweight subjects or after acute exercise in our non-exercisers were still less than half of values reported in sedentary lean adults (27, 51, 60, 76). Therefore, while exercise is clearly an important therapeutic tool for reducing metabolic disease risk among overweight-to-mildly obese adults, important additional benefits may be gained from weight loss or additional exercise.

The reason why the single session of exercise did not enhance insulin action in our habitually active participants is unclear. Perhaps the exercise stimulus in our study was below some “threshold” required to further enhance insulin action the next day in these subjects who exercise regularly. Factors determining the magnitude of an exercise stimulus that may be required to improve insulin sensitivity are not clear, but certainly the intensity, duration, and energy expended during exercise are likely candidates. Exercise intensity is a major determinant for the magnitude of muscle glycogen used during exercise (87), and as described above, the exercise-induced reduction in muscle glycogen concentration is often observed along with the increased insulin sensitivity after exercise (108). Importantly, because habitual exercise training is known to markedly reduce the contribution of muscle glycogen use during exercise (50, 87), this may help explain the lack of an acute effect of exercise on ISI in our habitually active subjects. The fact that the single exercise session performed in our study provided a considerably lower exercise stimulus than what our participants who regularly exercise reported to perform on a regular basis may also contribute to our finding that acute exercise did not increase ISI in this regularly exercising cohort. The acute 1h session brisk walking in our study was equal to approximately 4.5 METs, whereas on average our active subjects reported participating in an hour of exercise at an intensity equal to over 6 METs. Therefore, an exercise stimulus closer to, or perhaps even greater than, that performed regularly may be required to further enhance insulin action in a group of overweight-to-obese adults who exercise routinely. This notion is generally consistent with the progressive overload principle (41) indicating that a progressive increase in “stress” must be provided to induce physiological changes/adaptations. It is also important to note that because our assessment of insulin sensitivity was based on established calculation using plasma glucose and insulin concentrations during and OGTT (69) rather than more sensitive measures, like a hyperinsulinemic-euglycemic clamp (21), it is possible we were unable to detect a relatively subtle improvement in insulin sensitivity the day after an acute session of moderate-intensity exercise in our cohort of regular exercisers.

Pro-inflammatory cytokines, including: IL-1 β , IL-6, TNF- α , and MCP-1, are associated with insulin resistance (78, 90), and plasma concentrations of these cytokines are often found to be elevated in overweight and obese adults (55, 67). Weight-loss is consistently found to lower the systemic abundance of many of these pro-inflammatory cytokines in conjunction with

improvements in insulin sensitivity (29, 61). Similarly, when exercise training is accompanied by weight loss the improvement in insulin sensitivity has been found to parallel the reduction in systemic inflammatory markers (24, 32). However, the effect of exercise without weight loss is less clear. Although plasma concentrations of most of the systemic inflammatory markers we measured were not affected by exercise training or acute exercise in our study, IL-1 β tended to be lower in our subjects who were habitual exercisers. Importantly, the exercise-induced improvement in ISI we found the day after the acute session of exercise in Non-Ex was related to the change in plasma IL-1 β concentration. IL-1 β is a pro-inflammatory cytokine primarily produced and secreted by monocytes, macrophages, and neutrophils. IL-1 β secretion is elevated in obesity, and accumulating evidence implicates IL-1 β in the pathogenesis of insulin resistance (36, 70, 77, 96). It has been suggested that IL-1 β may impair insulin action in skeletal muscle by activating other pro-inflammatory signaling pathways such as the Nuclear factor kappa-B (NF- κ B) pathway (2), which is known to reduce insulin signaling (48). The effects of exercise on IL-1 β are complex. A one-year intensive exercise training intervention involving both endurance- and resistance-type exercise has been found to lower plasma IL-1 β concentration in obese patients, independently of changes in weight or body fat (7). Our finding that the trend for a lower plasma IL-1 β in our habitual exercisers vs. non-exercising overweight adults did not reach statistical significance suggests that perhaps the regular exercise may need to be rather intensive in order to see a more robust effect on plasma IL-1 β concentration, as suggested by Balducci, et al, (7). Acute exercise also has important effects on IL-1 β . In lean healthy adults, plasma IL-1 β concentration has been found to increase during a session of exercise in lean, healthy adults – and then return to pre-exercise levels soon after exercise (and remains at basal levels in the days after exercise) (91). But our finding that acute exercise lowered plasma IL-1 β the day after exercise in our group of non-exercising overweight subjects suggests the effects of acute exercise in overweight adults who already exhibit elevated plasma IL-1 β may be different, and this lowering of IL-1 β may contribute to their exercise-induced improvement in ISI. This is in agreement with findings from a recent study in obese rodents in which a single session of exercise reduced IL-1 β mRNA expression in adipose tissue (in both adipocyte and stromal vascular fractions), with a resultant decline in plasma IL-1 β concentration that was accompanied by an enhanced systemic insulin sensitivity (79). Therefore, although causality cannot be determined in the current study, our results support the growing body of literature highlighting the role of pro-inflammatory cytokines, such as IL-1 β , in

the pathogenesis of insulin resistance among overweight adults as well as a contributing mechanism by which exercise leads to an improvement in whole body insulin sensitivity.

Although exercise training and a higher cardiorespiratory fitness (i.e., VO_2max) are both commonly linked with reduced risk for cardiovascular disease (47, 62, 63, 106), we found subjects blood lipid profile and blood pressure to be similar between our exercisers and non-exercisers. While many studies report that exercise training can improve blood lipid profile and lower blood pressure (25, 107), others do not (15, 95). Even in studies that report exercise-induced improvement, these changes are often very modest (53, 68). For example, meta-analysis from randomized control trials indicate that on average exercise training lowered LDL-C concentration by only 0.16 mmol/L and increased HDL-C by only 0.04 mmol/L (39). Additionally systolic and diastolic blood pressure appear to only be reduced by < 5 mmHg with exercise training (53). More consistent improvements in blood lipids and blood pressure are observed in individuals who show signs of dyslipidemia and hypertension (13, 18), but these improvements are still rather modest (e.g., 2 mg/dL increase in HDL-C and 5-7mmHg reduction in systolic blood pressure (25, 65)). Importantly diet-induced weight loss promotes robust and consistent improvements in blood lipids, blood pressure and other markers of cardiovascular disease risk (52, 68). These findings further emphasize that exercise without weight loss may not be an optimal therapeutic approach for improving blood lipids and blood pressure in overweight adults. It is possible that many of the improvements in cardiovascular risk factors reported in response to regularly performed exercise may stem from the most recent session of exercise. For example, a single session of exercise has been consistently found to lower blood pressure (albeit slightly; systolic: 6-10mm Hg, diastolic: 2-5 mmHg) in just the first few hours after exercise (74, 86, 93). However, this effect appears to be fairly short lived, lasting 4-6 hrs in normotensive individuals (104) and 10-12 hrs post-exercise in hypertensive individuals (83). Acute exercise has also been shown to produce meaningful reductions in plasma triglyceride levels, yet this appears to be largely dependent on higher energy expenditure associated with prolonged exercise (3, 19). In contrast, cholesterol levels appear to be largely non-responsive to a single session of exercise regardless of exercise intensity or energy expenditure (37, 109). Collectively our findings suggest that neither acute nor regular exercise induced robust improvements in these markers of cardiovascular risk among overweight, but otherwise, healthy adults. In combination with previous findings demonstrating little if any

improvement in cardiovascular disease risk with physical activity/exercise a more therapeutic approach for reducing cardiovascular disease risk among overweight adults may be to promote weight loss especially considering that modest reductions in weight are associated with clinically relevant improvements in health within this population (59).

Assessing the direct contribution of regular exercise on important clinical outcomes like insulin resistance, blood lipids and blood pressure can be very challenging. For example, because a minor reduction in body weight can markedly increase insulin sensitivity (59) if exercise is accompanied by even a subtle degree of weight loss this would confound the interpretation of the underlying cause for the improvement in insulin resistance. Along these lines, reports of improvements in insulin action resulting from an exercise training program that persisted for over two weeks after the last exercise training session (6) may actually be due to participants' slight, yet significant weight loss. Moreover, an acute negative energy balance (i.e., energy expenditure exceeds energy intake) that may result from a more physically active lifestyle can also acutely improve insulin sensitivity (4) independently of the effects of an increase in physical activity. Additionally even modest weight loss can have clinically meaningful reductions in plasma lipids and blood pressure (59). Therefore, in these conditions it is often impossible to differentiate the effects of this weight loss and/or energy imbalance from the effects of the increased physical activity. Although our cross-sectional study design certainly has limitations, we were very strict about weight stability among all of our subjects, and we tightly matched key phenotypic characteristics of our group of regular exercisers and non-exercisers (e.g., body weight, body composition, waist circumference), which increases confidence regarding the independent effects of regular exercise on ISI and the other clinical outcomes that we reported here. Understanding the independent effects of exercise behavior (separate from the effects of weight loss and negative energy balance) on important health outcomes is very valuable in the development of lifestyle programs aimed at improving metabolic health.

The overall objective of this study was to help distinguish between the cardiometabolic health benefits associated with habitual exercise and those stemming from the most recent session of exercise in weight-stable overweight-to-mildly-obese adults. We found that engaging in a regular exercise routine was associated with a persistently elevated insulin sensitivity index in overweight adults, but not with a reduction in other key cardiometabolic disease risk factors (i.e., blood lipids

or blood pressure). While the mechanism(s) underlying the persistent effect of regular exercise on insulin sensitivity among overweight adults is not clear, alterations in muscle lipid metabolism as well as adaptations in tissues other than muscle (i.e., liver and adipose) may be contributing. In response to a single session of moderate-intensity exercise, insulin sensitivity index improved the day after exercise in overweight adults who do not normally exercise, but not in those who exercise regularly. As a result the difference in insulin sensitivity between our group of regular exercisers and non-exercisers was largely eliminated. The exercise session lowered plasma IL-1 β concentration in the habitually sedentary overweight subjects, suggesting a reduction in systemic inflammation may contribute to their enhanced insulin action after exercise. Therefore, while habitual exercise is associated with enhanced insulin sensitivity among overweight adults, just one session of exercise was enough to increase insulin sensitivity among sedentary overweight adults to levels equal to regular exercisers.

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Table 4-1. Baseline participant characteristics

| | EX | Non-EX |
|---------------------------------------|-------------------|--------------------|
| Sex (M/F) | (7/9) | (4/8) |
| Age (y) | 26.2 ± 1.3 | 28.1 ± 1.9 |
| Body weight (kg) | 89.4 ± 2.7 | 88.4 ± 2.5 |
| BMI (kg/m ²) | 30.5 ± 0.8 | 30.0 ± 0.5 |
| Body fat (%) | 33.5 ± 1.4 | 36.8 ± 2.0 |
| Fat mass (kg) | 30.2 ± 1.9 | 32.4 ± 1.8 |
| Fat free mass (kg) | 59.2 ± 1.8 | 56.0 ± 2.5 |
| Fasting glucose (mmol/L) | 4.3 ± 0.1 | 4.6 ± 0.1 |
| Fasting insulin (uU/mL) | 17.6 ± 1.5 | 22.3 ± 2.6 |
| VO ₂ max (ml/kg/min) | 32.9 ± 2.8 | 24.2 ± 1.7* |
| Planned exercise (MET-hours per week) | 32.3 ± 2.6 | 0.0 ± 0.0* |

Values are expressed mean ± SE

* Significantly different than EX, P < 0.05

Table 4-2. Oxygen consumption and heart rate during the exercise session

| | EX | Non-EX |
|---|-------------|-------------|
| VO ₂ during exercise (L/min) | 1.6 ± 0.2 | 1.3 ± 0.1 |
| VO ₂ max (%) | 53.5 ± 2.5% | 60.3 ± 4.1% |
| Heart rate (bpm) | 140.1 ± 2.3 | 138.3 ± 1.8 |
| Predicted HRmax (%) | 72.1 ± 0.9% | 71.8 ± 0.7% |

Values are expressed mean ± SE

Table 4-3. Fasting plasma substrate, hormone, and lipid profile

| | EX | | Non-EX | |
|----------------------|------------|------------|-------------------|--------------------|
| | Day 1 | Day 2 | Day 1 | Day 2 |
| Glucose (mmol/L) | 4.3 ± 0.1 | 4.3 ± 0.1 | 4.6 ± 0.1 | 4.5 ± 0.1 |
| Insulin (uU/mL) | 17.6 ± 1.5 | 18.5 ± 1.2 | 22.3 ± 2.6 | 19.3 ± 2.7† |
| Total-C (mg/dL) | 148 ± 9 | 147 ± 8 | 153 ± 7 | 151 ± 6 |
| LDL-C (mg/dL) | 121 ± 10 | 121 ± 9 | 122 ± 8 | 121 ± 6 |
| HDL-C (mg/dL) | 27 ± 2 | 26 ± 2 | 31 ± 3 | 30 ± 3 |
| Triglyceride (mg/dL) | 111 ± 14 | 118 ± 15 | 84 ± 10 | 91 ± 11 |
| NEFA (µmol/L) | 407 ± 36 | 328 ± 28‡ | 418 ± 46 | 399 ± 42 |

Total Cholesterol, Total-C; Low-density lipoprotein cholesterol, LDL-C; High-density lipoprotein cholesterol, HDL-C. Values are expressed mean ± SE. † Significant difference between Day1 and Day 2 within Non-EX, P<0.05. ‡ Significant difference between Day 1 and Day 2 within EX, P < 0.05.

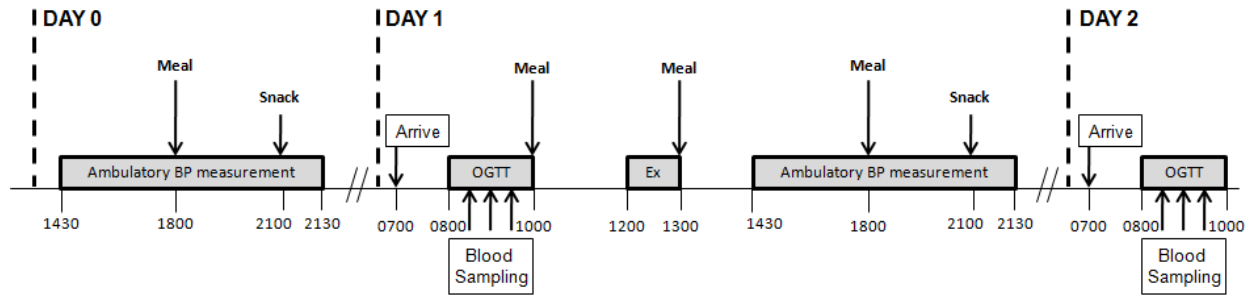


Figure 4-1. Timeline of experimental events.

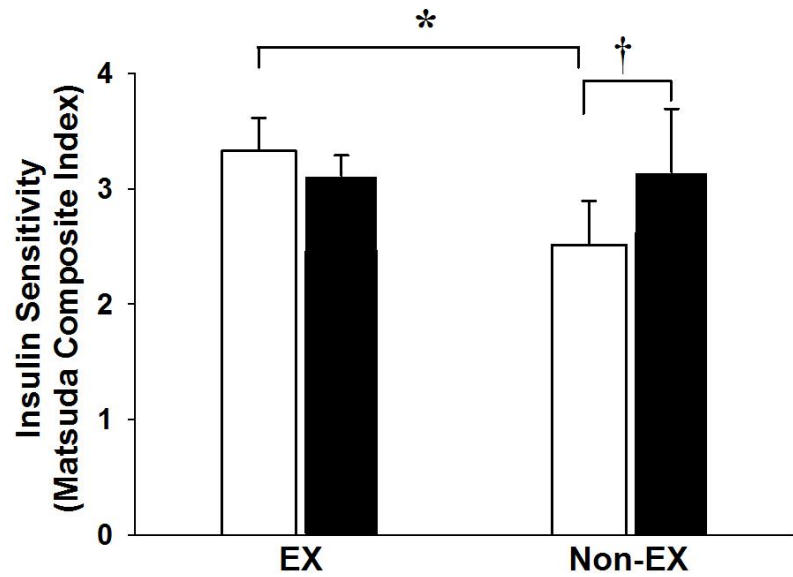


Figure 4-2. Insulin Sensitivity Index measured during an OGTT before (Day 1; open bars) and the day after a single session of exercise (Day 2; closed bars). * Significantly different than the EX cohort on Day 1, $P < 0.05$. † Significant difference between Day 1 and Day 2 within the Non-EX cohort.

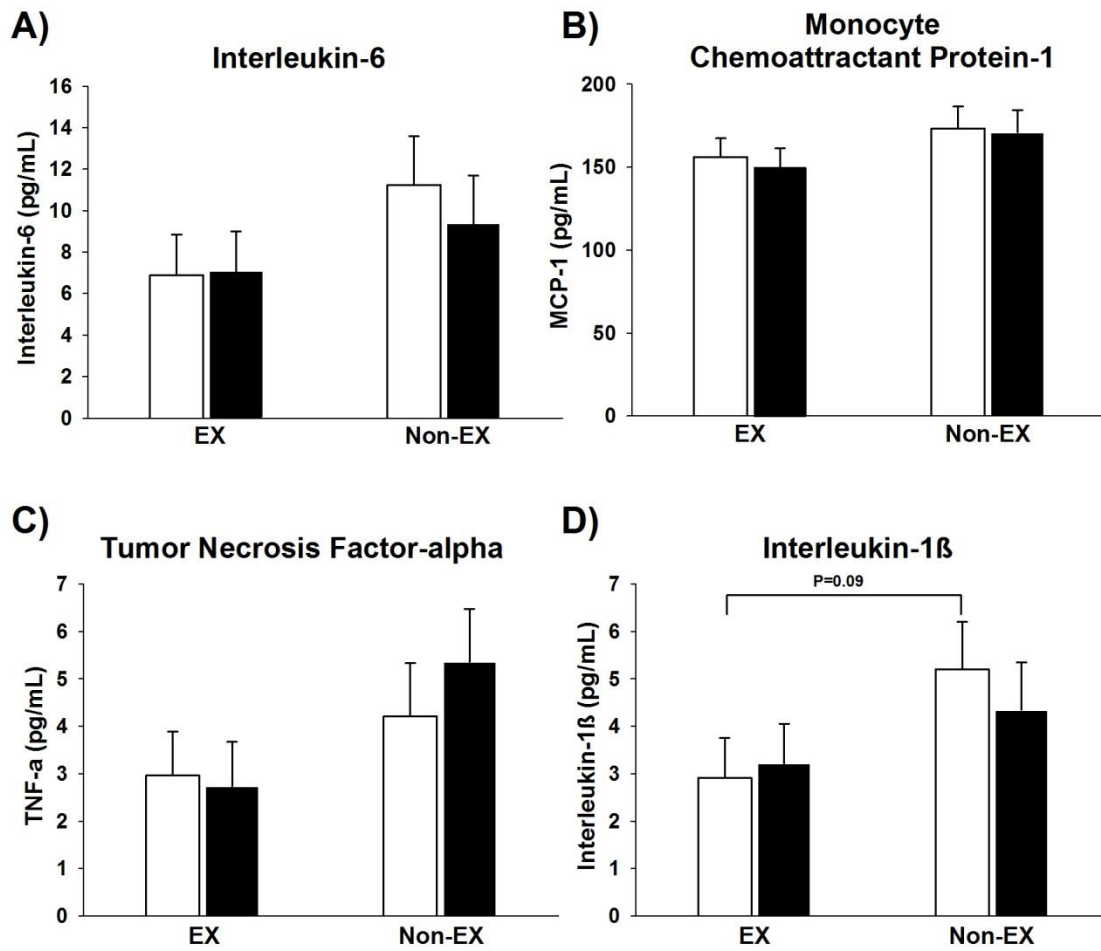


Figure 4-3. (A) Interleukin-6, (B) Monocyte Chemoattractant Protein-1, (C) Tumor Necrosis Factor-a, and (D) Interleukin-1 β concentration measured before (Day 1; open bars) and the day after a single session of exercise (Day 2; solid bars) in our EX and Non-EX participants.

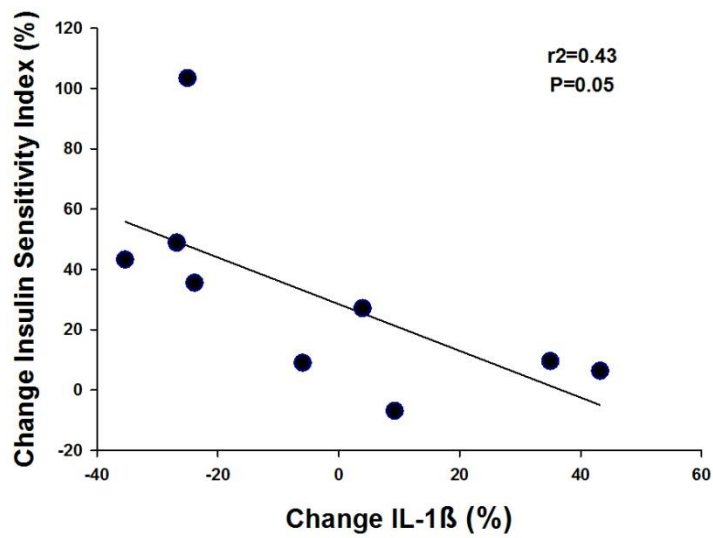


Figure 4-4. Relationship between percent change in fasting IL-1 β concentration and percent change in Insulin Sensitivity Index as measured during an OGTT using the Matsuda Composite Index before (Day 1) and the day after a single session of exercise (Day 2) in our Non-EX participants.

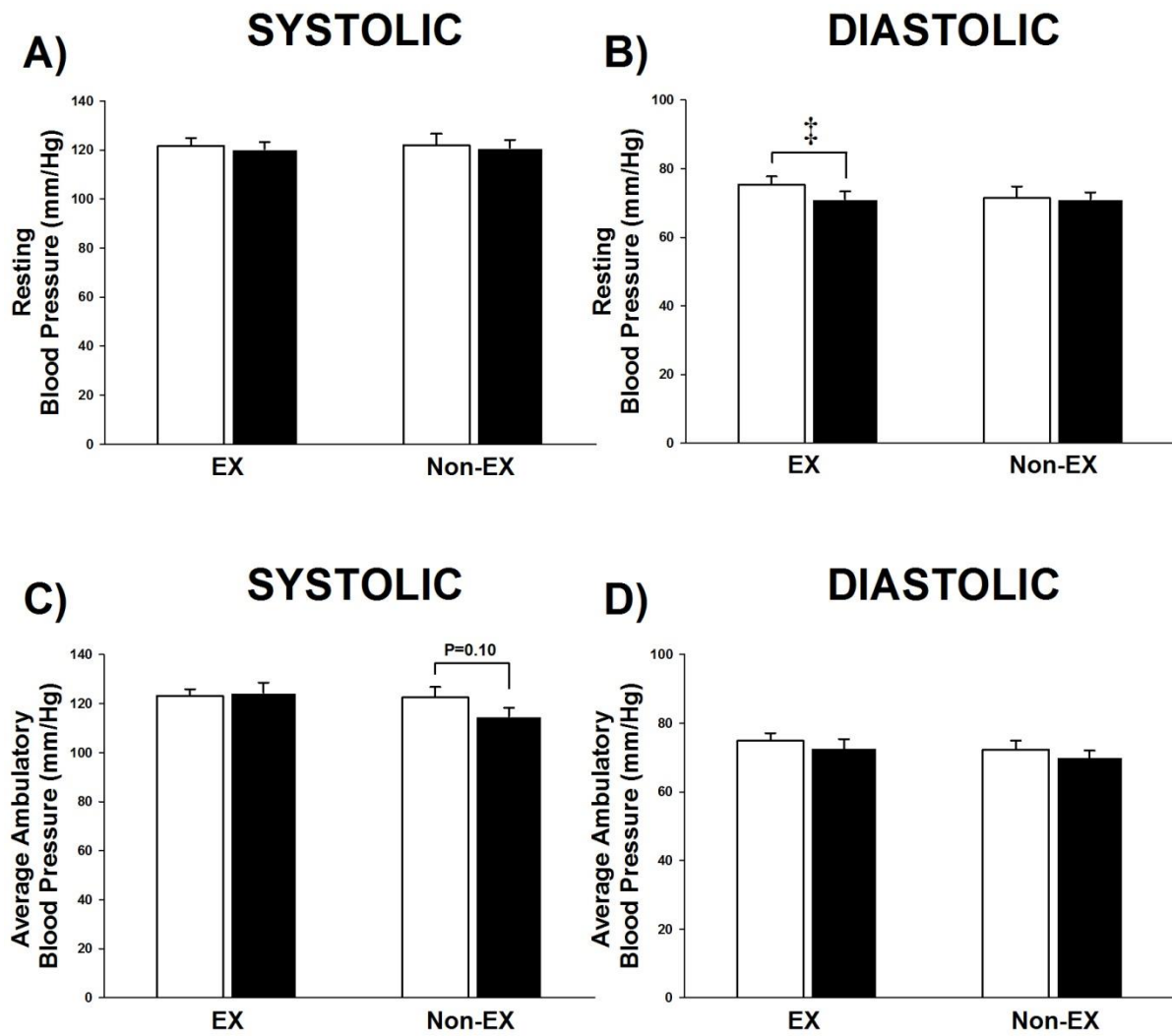


Figure 4-5. (A) Resting systolic, (B) resting diastolic, (C) ambulatory systolic, and (D) ambulatory diastolic blood pressure before (Day 1; open bars) and the day after a single session of exercise (Day 2; closed bars). ‡ Significant difference between Day 1 and Day 2 within EX group, $P < 0.05$.

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CHAPTER 5

Progressive metabolic adaptations to low intensity exercise training in obese adults

Abstract

The aim of this study was to examine the progressive adaptations that occur after 1 session, 2 weeks, and 3 months of low-intensity exercise in previously sedentary obese adults. Ten sedentary obese adults (men/women: 3/7; 38 ± 1 kg/m²; 30 ± 3 yrs) completed 5 experimental trials: 1) before beginning the exercise program (“PRE”), 2) after a single session of low-intensity exercise (40min at 60% HRmax), 3) after 2 weeks (6 days/wk) of this same exercise (“2WKS”), 4) again after 3 months of training (“3M”), and 5) exactly 3 days after their last session of the 3 month exercise training program (“3d after EX”). We performed a meal tolerance test 1h after exercise (or without prior exercise in “PRE” and “3d after EX”) to calculate the “insulin sensitivity index” (ISI) in the few hours after exercise, and we performed a hyperinsulinemic-euglycemic clamp the next morning ~16 hr after exercise (or without prior exercise in “PRE” and “3d after EX”). We also measured fatty acid oxidation, as well as total and LDL cholesterol. We found a single session of exercise did not significantly alter any of our primary outcome measures. However, by 2WKS ISI (2.1 ± 0.2 vs. 2.4 ± 0.2 ; $P=0.02$) and hepatic insulin resistance (33 ± 4 vs. 27 ± 2 ; $P=0.03$) were improved in the few hours after exercise. ISI did not improve further after 3 months of the exercise program. By 3 months of training, we observed a trend for an improvement in insulin sensitivity measured via the clamp the day after exercise, but this did not quite reach statistical significance (Glucose Rd/SSI: 2.4 ± 0.3 vs. 2.9 ± 0.3 (mg/min)/[SSI]; $P=0.065$). This trend for improved insulin sensitivity was no longer apparent 3d after EX. The exercise training program also increased fatty acid oxidation during exercise (8.6 ± 1.3 to 11.8 ± 1.7 $\mu\text{mol/kgFFM/min}$ ($P<0.01$)) and at rest (3.8 ± 0.2 to 4.5 ± 0.3 $\mu\text{mol/kgFFM/min}$ ($P=0.02$)) by 2WKS, and remained elevated throughout 3 months of training. Three months of training also significantly reduced Total-C (184 ± 11 vs. 163 ± 7 mg/dL) and LDL-C (152 ± 11 vs. 131 ± 9 mg/dL) (both $P<0.05$). Importantly, all adaptations were independent of any change in body mass (108 ± 4 vs. 108 ± 5 kg) or fat mass (52 ± 3 vs. 53 ± 4

kg). In summary, although a single 40 min session of exercise performed at 60%HRmax was not sufficient to induce any measurable changes in our markers of cardiometabolic health, we did find improvements in insulin sensitivity by 2 weeks as well as improvements in blood cholesterol by 3 months of this mild exercise training program in weight-stable obese adults.

Keywords: *Insulin resistance, glucose tolerance, physical activity*

Introduction

Most current exercise recommendations are designed with the goal to improve cardiorespiratory fitness (e.g. maximal oxygen uptake (VO_{2max})) (1). However, mounting evidence suggests many health benefits of exercise are largely independent of improvements in “fitness” (13, 22, 27). Along these lines, in PROJECT #1 of my dissertation we found that daily physical activity was a strong and significant predictor of insulin resistance, but VO_{2max} was not. These findings support the notion that a training program specifically designed to increase VO_{2max} may not be optimal for improving metabolic health. Moreover, exercise prescriptions that focus on enhancing VO_{2max} may actually be counter-productive because the higher intensity exercise often required to improve VO_{2max} has been associated with a greater incidence of injuries and participant discomfort leading to poor exercise adherence (23). In contrast to the relatively high intensity exercise required to improve VO_{2max} , profound reductions in cardiometabolic disease risk result from engaging in rather low or modest intensity physical activity (42).

Although adaptations to a physically active lifestyle can certainly improve important health outcomes, many cardiometabolic health benefits of exercise actually stem from the most recent exercise session(s), rather than from adaptations to weeks, months, and even years of exercise (22, 57, 61, 70, 72). In PROJECT #2 of my dissertation, although we did find that habitual exercise resulted in some persistent improvement in insulin sensitivity, we found a single session of moderate intensity exercise improved insulin sensitivity the next day in overweight-to-mildly-obese adults, which is in line with several previous reports (22, 57, 61). Our findings from PROJECT #2 also suggest that metabolic responses to an exercise session in overweight adults who do not exercise regularly are different from those who do exercise regularly. Therefore, adaptations that accrue as a non-exerciser begins to adopt a more physically active lifestyle appear to impact the metabolic response to each exercise session. However, the underlying mechanisms

that lead to improvements in health in the early stages of adopting a mild exercise training program remain unclear.

An important link between obesity and the development of insulin resistance (and many other metabolic diseases) is the overly-abundant mobilization of fatty acids in the circulation – and the subsequent delivery and uptake of excessive amounts of fatty acids into insulin-responsive tissues like skeletal muscle and liver. Consequently, this overabundance of fatty acid can result in the accumulation of fatty acid by-products in these tissues that can activate pro-inflammatory pathways, which in turn can induce insulin resistance (10). A previous study from our laboratory demonstrated that exercise can alter the metabolic fate of fatty acids that enter the muscle cell, thereby reducing the accumulation of lipid intermediates (e.g., DAG and ceramide) known to impair insulin signaling (74). However, this earlier study was performed in lean subjects who were exposed to an overnight lipid infusion after a session of vigorous exercise (74). More recent findings from our lab indicate that when obese subjects exercised at a relatively low intensity (50% VO_2max) the changes in muscle lipid metabolism were far less robust, and skeletal muscle pro-inflammatory stress was not reduced (61). This recent study (61) only examined the effects of a single session of low intensity exercise, and it is possible that at least a few successive sessions of exercise at these low intensities may be required to impart a measurable effect on muscle lipid metabolism and inflammatory pathway activation. Additionally, exercise-induced changes in inflammatory status within adipose tissue and the systemic circulation may contribute to improvements in insulin resistance as well as other cardiometabolic diseases risk factors (34, 62, 76). Importantly a better understanding of the progressive adaptations associated with adopting a low-intensity exercise training program on fatty acid metabolism, pro-inflammatory pathways, and ultimately on insulin resistance, could have important implications for designing lifestyle interventions aimed at reducing cardiometabolic disease risk in obese adults.

The overall aim of this study was to examine the progressive adaptations that occur after 1 session, 2 weeks, and 3 months of adopting a low-intensity exercise program in previously sedentary obese adults. We hypothesized that a single session of mild intensity exercise would not be sufficient to improve insulin resistance the next day. However, we did anticipate insulin resistance would improve after 2 weeks of exercise with no further improvement after 12 weeks. We predicted that

reductions in both the accumulation of fatty acid by-products and pro-inflammatory activation within muscle would parallel the improvement in insulin action. Finally, we hypothesize that insulin resistance would return to pre-training levels when participants abstain from exercise for only three days after training.

Methods

Subjects

A total of 10 obese males (n=3) and females (n=7) (body mass index [BMI]: 37 ± 1 kg/m²; age: 30 ± 3 yrs) were recruited to participate in this study. We performed a detailed health history, physical examination, as well as a 12-lead electrocardiogram, and standard blood and urine tests. Participants were not taking any medication known to influence the metabolic parameters we were measuring. All subjects were non-smokers, weight stable (i.e., ± 2 kg for ≥ 6 months), sedentary (no regularly planned exercise/physical activity), and had no health history of metabolic or cardiovascular diseases. Written, informed consent was obtained from all subjects before initiating participation. All procedures of this study were approved by the University of Michigan Institutional Review Board.

Preliminary testing

At least one week before their first experimental trial, participants completed an incremental test to exhaustion on a cycle ergometer to determine VO₂peak (a marker of cardiorespiratory fitness) and maximum heart rate (HR_{max}). The protocol for this test included a 4 minute warm-up followed by a progressive increase in intensity each minute until voluntary exhaustion (73). Body composition was also assessed using dual energy X-ray absorptiometry (DEXA; Lunar DPX DEXA Scanner).

Experimental protocol for each hospital visit

All subjects were admitted to the Michigan Clinical Research Unit (MCRU) at the University of Michigan Hospital a total of five times for metabolic testing throughout the ~3 month participation in the entire study (Figure 5-1). All metabolic studies were identical (Figure 5-2) with the exception that subjects did not exercise during visits #1 and #5.

The evening before each trial, subjects ingested a standardized meal at 1900h (one-third of total daily caloric requirements; 55% carbohydrate, 30% fat, and 15% protein). The next morning (Day 1), subjects were provided a standardized breakfast and lunch to be eaten at 0930h and 1230h, respectively (see details of all meals provided during the experiments in “Study Diets” section, below). At 1600h subjects exercised for 40 minutes at approximately 60% of their pre-determined HR_{max} (~50% VO_{2peak}) on a cycle ergometer. In order to quantify energy expenditure during exercise, we measured VO_2 and VCO_2 using a metabolic cart (PhysioDyne Technologies) within the first and last 10 minutes of the exercise session. All metabolic studies were identical with the exception that subjects did not exercise during visits #1 and #5. During the two non-exercise visits they remained seated quietly during this time. After the exercise period, subjects were allowed to shower and they were provided meals at 1800h and 2000h and an evening snack at 2200h (see details in “Study Diets” section, below). One intravenous catheter (IV) was placed in a hand vein for blood sampling, and a second IV was placed in a forearm vein for infusions that began the next morning. During all visits, a urine pregnancy test was performed on all female subjects when they arrived on the first day of the experiment. Other than the supervised exercise session, all subjects remained relatively sedentary in the hospital until completion of the trial the next day.

Beginning at 0450h the next morning (Day 2), 3 blood samples were taken in 5 min intervals (i.e.; 0450h, 0455h, and 0500h) from the heated hand vein to obtain “arterialized” blood samples (44), for determination of background enrichment of [6,6 d_2]glucose, [1- ^{13}C]-palmitate, and [1,1,2,3,3 d_5]-glycerol. A background sample was also used to determine fasting substrate and hormone concentrations as well as markers of systemic inflammation. At 0500h we began a primed, constant rate infusion of [6,6 d_2]glucose (35 $\mu\text{mol/kg}$ priming dose; 0.41 $\mu\text{mol/kg/min}$ continuous infusion) for measurement of hepatic glucose production and whole-body glucose uptake. We also measured resting metabolic rate (and fat oxidation) for 30 min starting at 0630h using a VMax Encore metabolic cart (SensorMedics). At 0700h we obtain a muscle sample (~100 mg) from the vastus lateralis. This muscle sample was cleaned with saline, dried, and then aliquots of this sample were quickly frozen in liquid nitrogen. Samples were stored at -80°C until analysis. In addition, we also obtained a 100-200mg subcutaneous adipose tissue sample from the abdominal region. Similar to the muscle samples, the adipose tissue biopsy was cleaned with saline, dried, and aliquots were quickly frozen in liquid nitrogen. Samples were stored at -80°C until analysis. At

0700 we also began a primed, constant-rate infusion of [1,1,2,3,3 d₅]-glycerol (1.5 μmol/kg priming dose; 0.10 μmol/kg/min continuous infusion). Then at 0800h we began a constant-rate infusion of [1-¹³C]-palmitate (0.04 μmol/kg/min continuous infusion). At 0845h, three arterialized blood samples were obtained from a heated hand vein in 5 min intervals for determination of glycerol rate of appearance (Ra) in plasma [used as an index of whole body lipolysis], fatty acid Ra and fatty acid rate of disappearance (Rd) from plasma [fatty acid mobilization and uptake, respectively], as well as determination of basal hepatic glucose production via isotope dilution of the constant rate infusion of [6,6 d₂]glucose. These blood samples were analyzed for plasma concentrations of glucose and insulin. At 0900h we began a hyperinsulinemic-euglycemic clamp to assess peripheral insulin sensitivity, as described previously by Dr Ralph DeFronzo's laboratory (58). Briefly, the clamp was performed using a primed 2h insulin infusion at a rate of 40 mU/m²/min. Plasma glucose concentration was monitored every 5 minutes during the clamp study using a glucose auto-analyzer (Yellow Springs Instruments), and glucose (D20 dextrose solution) was infused at a variable rate to maintain plasma glucose concentration at participants fasting blood glucose concentration (~5 mM). Importantly, this glucose infusion solution was enriched with [6,6 d₂]glucose (2.5% enriched) to limit changes in glucose tracer enrichment in plasma (40). In addition to the small blood samples collected every ~5 minutes to assess plasma glucose concentration, we also collect additional plasma samples for assessment of insulin and plasma enrichment of [6,6 d₂]glucose during the final 20 min of the 2h clamp. Subjects also received an intravenous infusion of potassium (KCl) during the clamp to prevent hypokalemia. After completing the clamp procedure, subjects were provided a meal, and were monitored until plasma glucose concentration was stable. When vital signs are stable, subjects will be discharged from the hospital.

Exercise training program

Subjects completed a 3-month mild-intensity, exercise training program. They were required to exercise 6 days each week for 40 min/day at an intensity eliciting 60% of their HRmax (~50% VO₂max).

Study Diets

The total energy content of the diet for each hospital stay was estimated to match the energy expended. Energy expenditure on each exercise day while admitted to the hospital was estimated as: $\{[(VO_2 \text{ during exercise}) \times (5\text{kcal/liter of } O_2 \text{ consumed}) \times 40\text{min}] + [597+(26.5 \times \text{fat free mass})]\}$ (71). Diets were designed to maintain "energy balance" (i.e.; energy intake = energy expenditure) during all trials in order to avoid any confounding influence of a negative energy balance on insulin sensitivity (4). 20% of daily energy intake was consumed at 0930h, 1230h, and again at 1800h. 30% of total daily energy requirement was provided at 2000h, and 10% of daily energy requirement was provided in the evening snack (2200h). After this snack, subjects did not consume anything but water until completion of the clamp procedure the next day.

During the 3-month exercise training program, it was very important to our study design that the subjects maintain their body weight. Subjects were asked to maintain their normal dietary habits, they weighed themselves daily and reported their daily body weight to research staff members. We also collected a 3 day dietary journal 3 days leading up to Visit #1, after 6 weeks of exercise training, and at the end of the 3-month exercise training program. If body weight changed $\pm 1\text{kg}$ during the program, dietary consultation was provided by our research dietitian to help maintain their original body weight.

Analytical Procedures

Plasma substrate and hormone concentrations

Blood samples were collected in chilled EDTA tubes, centrifuged (1,600 g for 20 min. at 4°C) within 30 min of collection, and then stored at -80°C until analysis. Plasma glucose (glucose oxidase assay; Fisher Scientific), fatty acid (NEFA-HR assay kit; WAKO Chemicals USA), triacylglyceride (Triacylglyceride reagent; Sigma Adrich), total- and high-density lipoprotein (HDL; Cholesterol E and HDL-Cholesterol E; Wako Chemicals USA) concentration were measured with commercially available colorimetric assay kits. Plasma insulin concentration was measured using a commercially available radioimmunoassay (RIA) kit (Human insulin RIA kit; Millipore).

Muscle and adipose tissue lysate preparation

Previously weighed frozen muscle and adipose tissue samples were homogenized in commercially available ice cold lysis buffer (Triacylglyceride reagent; Sigma Adrich) supplemented with commercially available protease inhibitors (Triacylglyceride reagent; Sigma Adrich) using a TissueLyser (Qiagen, Valencia, CA). Samples were then centrifuged at 1500g for 10 minutes at 4°C, the upper lipid layer was removed from adipose tissue samples and the supernatant (whole cell lysate) collected. Protein concentration was measured using a commercially available BCA protein assay kit (ThermoFisher Scientific, Rockford, IL).

Multiplex analysis

Skeletal muscle proteins associated with inflammation and insulin resistance (total NFκB, total IκBα, phosphorylated JNK, Akt^(serine473)) were measured using commercially available multiplex cell-signaling bead kits (c#:48602, Milliplex MAP Kit, Millipore, Billerica, MA). Adipose tissue and plasma cytokines associated with inflammation (TNF-α, MCP-1, VEGF, IL-6) were measured using commercially available multiplex bead kits (c#: HCYTOMAG-60K, Milliplex MAP Kit, Millipore, Billerica, MA). Samples were analyzed using the Luminex L200 instrument (Luminex, Austin, TX) and quantified by xPONENT software (Luminex).

Plasma fatty acid, glycerol, and glucose tracer-tracee ratio (for substrate kinetics calculations)

The plasma tracer-tracee ratio (TTR) for palmitate, glycerol, and glucose were determined by gas chromatography-mass spectrometry (GC/MS) with Mass Selective Detector (Agilent Technologies) as previously described (66). Briefly, starting with 250 μL of plasma and 250 μL of internal standard (C17:0), proteins were precipitated from plasma with acetone and lipids extracted with hexane. The hexane fraction was collected and iodomethane was used to convert fatty acids to methyl esters (FAMES) that were isolated by solid-phase extraction columns (Sigma Aldrich). Following electron impact ionization, selectively monitoring mass-to-charge ratios of ions 74 and 75 were used to determine enrichment of methyl ester palmitate. Plasma glycerol and glucose were isolated from the aqueous portion of the deproteinized sample. The aqueous fraction was dried down and reconstituted in 100 μL of dH₂O. A 25 μL aliquot was extracted to create a glucose derivative after drying it down, reconstituting it in 2% hydroxylamine in pyridine (50μL) followed by a 20 minute incubation at 100°C. Acetic Anhydride (50 μL) was added to the sample

and incubated for 40 minutes at 100°C. Samples were then diluted in ethyl acetate and stored at -20°C until GC/MS procedures. Following electron impact ionization, selectively monitoring mass-to-charge ratios of ions 187 and 189 were used to determine enrichment of [6,6 d₂] glucose. The remaining 75 µL was dried down, reconstituted in pyridine (25 µL) and acetic anhydride (25 µL), and incubated for 60 minutes at room temperature to create the glycerol derivative. Following electron impact ionization, selectively monitoring mass-to-charge ratios of ions 145 and 148 were used to determine enrichment of [1,1,2,3,3 d₅]-glycerol.

Intramyocellular glycogen concentration

Muscle glycogen was determined from measurement of muscle glucose after acid hydrolysis in previously weighed and dried muscle samples (65). Briefly, muscle samples were homogenized in dH₂O and 2N HCl acid added to neutralize enzymatic activity. After a 1 hr incubation at 100°C samples were neutralized with 1N NaOH to a pH of 6.5-7.5. Free glucose concentration was determined as previously described (65).

Intramyocellular lipid species concentration

Frozen muscle samples were rapidly homogenized in ice-cold saline and lipids then extracted in a single-phase mixture of chloroform-methanol-saline (1:2:0.8) (9) and internal lipid markers for IMTG, DAG, and non-esterified fatty acid (NEFA) with fatty acid moieties of odd carbon number were added for subsequent purity and recovery determinations (NuChek, Elysian, MN; Avanti Polar Lipids, Alabaster, AL). After brief centrifugation, the lower chloroform phase containing lipids was transferred to a clean tube and dried under vacuum. 15% ethyl acetate (EtOAc) in hexane added to the lipid residue to dissolve diacylglycerol (DAG), triacylglycerol (TAG) and was eluted from extraction tube containing 500 mg normal phase silica and was dried down. Samples were again reconstituted in 15% ethyl acetate (EtOAc) in hexane and purified using thin-layer chromatography. Fatty acid methyl esters were generated from purified glycerolipids by alkaline methanolysis, a transesterification process as previously described (11), while NEFA were converted to methyl esters by a methyl iodide procedure (66). FAMES were measured by gas chromatography and electron-impact mass spectrometry (Agilent 6890A GC and 5973N MSD, Palo Alto, CA). Following electron impact ionization, selectively monitoring mass-to-charge ratios of ions 74, 79 and molecular ions) at retention times for individual FAMES, and quantified

using FAME standards (NuChek, Elysian, MN). A complete description of the muscle lipid analysis can be found in APPENDIX 3.

Western blotting

Previously weighed frozen muscle samples were homogenized in commercially available ice cold lysis buffer (Triacylglyceride reagent; Sigma Adrich) supplemented with commercially available protease inhibitors (Triacylglyceride reagent; Sigma Adrich), centrifuged at 1500g for 10 minutes at 4°C, and the supernatant (whole cell lysate) collected for protein concentration and western blotting analysis. SDS-page was used to separate 20µg of sample that was transferred to a nitrocellulose membrane. Blots were probed with the following antibodies: pIRS-1 Ser³¹² (catalog #: 2381S; Cell Signaling Technology), Complex-1 (catalog #: 459100; Invitrogen), CPT-1 (catalog #: sc20514; Santa Cruz Biotechnology), CD36 (catalog #: sc9154; Santa Cruz Biotechnology), GPAT (a gift from RA Coleman), DGAT-1 novus (catalog #: NB110-41487; Novus Biologicals), and DGAT-2 (catalog #: sc66859; Santa Cruz Biotechnology). Nitrocellulose membranes were incubated in species specific secondary antibodies, developed with enhanced chemiluminescence (Amersham Biosciences), and quantified by densitometry (Alpha Ease FC, Alpha Innotech Corp.). Within subject comparisons were prepared on the same blot.

Calculations

Indices of Insulin Sensitivity: Plasma glucose and insulin concentrations measured before and during the meal tolerance test were used to assess whole body insulin sensitivity using the Matsuda Composite Index and hepatic insulin resistance as previously described (2).

$$\mathbf{Matsuda\ Composite\ Index} = 10000/\sqrt{[(fasting\ Insulin\ x\ fasting\ glucose)\ x\ (2h\ Insulin\ x\ 2h\ glucose)]}$$

$$\mathbf{Hepatic\ Insulin\ Sensitivity\ Index} = [Glucose\ AUC_{0-30}] \times [Insulin\ AUC_{0-30}]$$

Hepatic glucose production (HGP). Basal HGP (before the hyperinsulinemic-euglycemic clamp) was calculated using the equation of Steele for steady-state conditions [$HGP_{basal} = Glucose\ Ra =$

([d₂]-glucose infusion rate)/TTR] (82). During the hyperinsulinemic-euglycemic clamp, total glucose Ra was calculated using the Steele equation (82) from samples collected during the steady-state period of the clamp (i.e., last 20 min). HGP during the clamp was calculated as the difference between Glucose Ra during the clamp and exogenous glucose infusion rate (GIR) used to maintain euglycemia during the steady state period of the clamp ($HGP_{\text{clamp}} = \text{Glucose Ra} - \text{GIR}$).

Rate of glucose disappearance from plasma (Glucose Rd). Under basal conditions (before the hyperinsulinemic-euglycemic clamp), $\text{Glucose Rd}_{\text{basal}} = \text{Glucose Ra}_{\text{basal}}$ (or HGP_{basal}), so $\text{Glucose Rd}_{\text{basal}}$ was also be calculated using the Steele equation for steady state conditions (82). During the clamp, steady-state Glucose Rd was calculated using the Steele equation (82) from samples collected during the steady-state period of the clamp (i.e., last 20 min).

Insulin sensitivity. Hepatic insulin sensitivity was calculated as the insulin-induced percent suppression of endogenous glucose production.

$$\text{Hepatic insulin sensitivity} = [1 - (HGP_{\text{clamp}}/HGP_{\text{basal}})] \times 100\%$$

Peripheral insulin sensitivity was calculated as the total rate of glucose uptake during the steady state portion of the clamp ($\text{Glucose Rd}_{\text{clamp}}$), normalized to the steady-state insulin concentration during the clamp (steady-state $\text{insulin}_{\text{clamp}}$).

$$\text{Peripheral insulin sensitivity} = \text{Glucose Rd}_{\text{clamp}} / (\text{steady-state } \text{insulin}_{\text{clamp}})$$

Rate of fat oxidation: Whole body fat/triacylglycerol oxidation (g/min) was calculated from VO_2 and VCO_2 measurements using the equations of Frayn (29). Whole body fatty acid oxidation was calculated by dividing triacylglycerol oxidation by an estimated molecular weight of triacylglycerol (860 g/mol) and multiplying by 3.

Glycerol and fatty acid kinetics. We used the Steele equation for steady-state conditions (82) to calculate both glycerol Ra (assessment of whole-body lipolysis) and palmitate Ra (assessment of fatty acid mobilization into plasma). Because our measurements was performed under steady-

state conditions, palmitate Ra = palmitate Rd, which provides an assessment of systemic fatty acid uptake. We also estimated total fatty acid Ra (and Rd) by dividing palmitate Ra (and Rd) by the ratio of plasma palmitate to total plasma fatty acid concentration. Fatty acid and glycerol kinetics were only measured in the basal state (i.e., non-insulin stimulated conditions)

Low-density lipoprotein cholesterol (LDL-C). LDL-C was calculated from measurements of plasma concentrations of total cholesterol (Total-C), HDL-C, and triacylglyceride (i.e., [LDL-C] = ([Total-C] – [HDL-C] – [Triacylglyceride])/5 (60))

Statistical analysis

Linear mixed model with time as a categorical factor was used to determine differences in outcome variables compared with measurements made before the exercise program (“PRE”).

Statistical significance was defined as $P \leq 0.05$.

Results

Compliance, fitness, and body composition responses to exercise training

Adherence to the prescribed exercise training program was very high. Subjects completed 99±2% of all exercise sessions during the first two weeks and 92±2% of all exercise sessions throughout the remaining 10 weeks of the exercise training program (see Appendix 3). Despite this very high compliance, our low-intensity exercise training program did not significantly increase VO₂max (Table 5-1, P=0.14) and our markers of mitochondrial density in skeletal muscle (i.e., complex-1 and carnitine palmitoyl transferase-1 [CPT-I]) were also not affected (data not shown). As designed, body weight and fat mass remained very stable throughout the 3-month exercise program (Table 5-1). This successful control of body weight and body composition allows for interpretation of these findings to focus specifically on the effects of the mild exercise training program.

Measures of insulin sensitivity

Meal tolerance test performed in the few hours after exercise. On Day 1 of each experimental trial we estimated whole-body insulin sensitivity index (ISI) using the Matsuda composite index during a meal tolerance test that began 80 minutes after completion of exercise (or rest during Visit #1 and #5). A single session of exercise did not affect ISI, but ISI was modestly, yet significantly

improved after 2 weeks of the exercise program (Figure 5-3A; $P=0.02$). This improvement in whole-body ISI at 2 weeks was accompanied by a significant reduction in hepatic insulin resistance (Figure 5-3B; $P=0.03$), as assessed by calculations previously established by Abdul-Ghani, et al., (2). ISI did not improve any further after 3 months of the exercise program (Figure 5-3A).

Hyperinsulinemic-euglycemic clamp performed the day after exercise. To assess more persistent effects of exercise on insulin sensitivity (i.e., into the next day), a hyperinsulinemic-euglycemic clamp was performed ~16h after exercise. We found insulin sensitivity to be largely unaffected the day after 1 exercise session or even after 2 weeks of exercise (Figure 5-4A). We did observe a trend for an improvement in insulin sensitivity after 3-months of this low-intensity exercise training program, but this did not quite reach statistical significance (Glucose Rd/SSI: 2.4 ± 0.3 vs. 2.9 ± 0.3 (mg/min)/[SSI]; $P=0.065$). This trend for improved insulin sensitivity at the end of the 3-month training program disappeared when the subjects abstained from exercise for 3 days (Figure 5-4). Similar to the trend for improved insulin sensitivity after 3 months of training, GLUT4 protein abundance also tended ($P=0.10$) to be elevated at this time, and this trend persisted even after the subjects did not exercise for 3 days. (Figure 5-4 C). Hepatic insulin resistance was remarkably stable after 1 session, 2 weeks, and 3 months of training (Figure 5-4B).

Skeletal muscle inflammation and markers of insulin signaling

In agreement with our finding that the exercise program did not robustly improve insulin action, markers of pro-inflammatory signaling in skeletal muscle, NF κ B, I κ B α , and pJNK were also unchanged after 1 session, 2 weeks or 3 months of the exercise training program (Table 5-2). Similarly, we did not find any changes in the phosphorylation state of Akt^{Ser473} or IRS-1^{Ser312} (Table 5-3), which have been identified as being crude markers of insulin signaling in non-insulin stimulated tissue (17, 53)

Skeletal muscle glycogen and lipid content

Muscle glycogen concentration did not change significantly throughout the 3 month training period (Figure 5-5A). Similarly, neither IMTG nor intramyocellular DAG content were significantly affected the day after 1 session, 2 weeks, and even after 3 months of this mild exercise training program (Figure 5-5 B-C), but there was a considerable degree of variability in these

muscle lipid measures. Abundance of key enzymes involved in IMTG synthesis (i.e., GPAT, DGAT1, and DGAT2) were also unchanged during the 3 month exercise training program (Table 5-4).

Changes in substrate oxidation and fatty acid uptake

The mild exercise training program did induce some changes in fuel selection both during exercise and at rest. Compared with fat oxidation measured during the very first exercise training session, 3 months of training significantly increased the mean exercise fatty acid oxidation from 8.6 ± 1.3 to 11.8 ± 1.7 $\mu\text{mol/kgFFM/min}$ ($P < 0.01$). Additionally, compared with before starting the training program, 2 weeks of exercise training increased resting fatty acid oxidation and it remained elevated after 3 months of exercise training (Figure 5-6 A). Accompanying this increase in resting fatty acid oxidation, we found a strong trend ($P = 0.12$) for an increase in fatty acid Rd (i.e., fatty acid uptake) starting at two weeks of the training program (Figure 5-6 B). This was also accompanied by a significant increase in skeletal muscle abundance of the fatty acid transporter FAT/CD36 after 3 months of exercise training (Figure 5-6 C). However, as noted above, we did not find changes in mitochondrial fatty acid transport protein CPT-1 (data not shown). Lipolytic rate (i.e., glycerol Ra) and re-esterification remained largely unaffected by the exercise training program (Table 5-5). We found a slight increase in fasting plasma fatty acid concentration after 2 weeks and 3 months of training (Table 5-6), which likely reflected an increase in lipolytic rate during exercise during the previous evening.

Blood lipid concentrations

The exercise program induced a progressive reduction in plasma total cholesterol (Total-C), which reached statistical significance after 3 months of training (Table 5-6, $P < 0.01$). This reduction in Total-C was due to a reduction in low-density lipoprotein cholesterol (Table 5-6, $P < 0.01$). The exercise program did not alter fasting triglyceride concentration (Table 5-6).

Markers of adipose tissue and systemic inflammation

Three months of low-intensity exercise training did not influence markers of adipose tissue inflammation including TNF- α , MCP-1, and VEGF (Table 5-7). Additionally systemic pro-

inflammatory proteins including IL-6, TNF- α , and MCP-1 did not change throughout the 3 month mild exercise training program (Table 5-8).

Discussion

Epidemiological evidence clearly indicates that even a rather modest increase in regular physical activity behavior in formerly sedentary adults can profoundly reduce disease risk and all cause mortality (12, 85). Yet the time course for physiological adaptations, and resultant changes in markers of cardiometabolic health during the early stages of adopting a physically active lifestyle are not clearly understood. Our major findings indicate that a single session of low-intensity exercise (i.e., the very first exercise training session) was not enough of a stimulus to improve insulin sensitivity, but our measures of whole-body insulin sensitivity and hepatic insulin resistance were improved after two weeks of the exercise program. The insulin sensitizing effects of exercise were relatively short-lived, as evidenced by improvements in insulin sensitivity when measured in the few hours after exercise but not the next morning. These improvements in insulin sensitivity within the few hours after exercise were maintained, but not enhanced further with an additional 10 weeks of exercise training. We also found that 3 months of low-intensity exercise training induced clinically meaningful improvements in the blood lipid profile of our obese subjects. Importantly, the beneficial health effects of exercise in this study could not be attributed to changes in body weight or body composition because these remained unchanged throughout the 3-month exercise program.

The precise mechanisms responsible for the improvement in the insulin sensitivity index (ISI) measured a few hours after exercise are not clear, but improvement in hepatic insulin resistance may be an important contributor to this effect – at least in the short-term. This is based on our observation that enhanced whole-body ISI after two weeks of the exercise program was accompanied by an improvement in our assessment of hepatic insulin resistance. Our finding that hepatic insulin resistance improved ~15% after 2 weeks of the exercise program, was similar to the improvement found in comparable cohort of obese subjects after 7 days of a more intense exercise training program (i.e., 1h of exercise at 85% HRmax for 7 consecutive days) (48). How the cumulative effects of a few sessions of exercise (without weight loss) may alter insulin action in the liver remains unclear. It has been suggested that regular exercise may lower or prevent

accumulation of lipid within the liver by suppressing lipogenic potential during the exercise session, as well as during the first few hours after exercise (45). Indeed seven continuous days of exercise training has been found to lower hepatic triglyceride content in obese adults with non-alcoholic fatty liver disease (36). Because hepatic lipid accumulation can negatively impact hepatic insulin action (16, 50, 80), perhaps even just a few days of exercise may be enough to induce a subtle, yet physiologically meaningful reduction in lipid accumulation in the liver that may translate to an improvement in hepatic insulin sensitivity. Interestingly, although the improvement in whole-body ISI was maintained throughout the next 10 weeks of our exercise program, the improvement in hepatic insulin resistance appeared to largely reverse. The reason why the improvement in hepatic insulin resistance may dissipate as the exercise program continued is puzzling, but if true, this suggests that the persistent improvement in whole-body ISI after 3 months was a consequence of a shift from improved hepatic insulin sensitivity in the early stages of the exercise program to enhanced peripheral insulin action.

Exercise training is often reported to improve insulin sensitivity in obese adults when measured several hours or even a few days after the most recent session of exercise (6, 20, 25, 27, 35, 41, 84). Unfortunately, many studies that examine the effects of exercise training on insulin sensitivity in obese adults do not strictly control body weight during the exercise program (6, 25, 41), making it impossible to differentiate the effects of the exercise training from the potent effects of weight loss. However, some studies designed to prevent changes in body weight during the training program have reported a persistent improvement in insulin sensitivity with exercise training without weight loss (27, 84), but this finding is not universal (28, 78). Findings from PROJECT #2 of my dissertation support the notion that exercise training in overweight adults may indeed induce adaptations resulting in a persistent improvement in insulin sensitivity (see Chapter 4). In contrast, in the present study we did not find a persistent improvement in insulin sensitivity after 3 months of training, especially when measured 3 days after the most recent session of exercise. Perhaps this discrepancy can be explained by the relatively low exercise stimulus used in our present study, which may have been insufficient to induce persistent effects on insulin action. Interestingly, we did find a strong trend for enhanced insulin sensitivity measured 16 hours after the last exercise training session of the 3 month exercise program. This modest improvement was largely driven by 6 out of our 9 participants, and these subjects increased their insulin sensitivity

by 15-57%. We acknowledge that the relatively small sample size likely contributed to our inability to detect a significant improvement in insulin sensitivity at 3 months when measured the day after the last session of exercise. However, it is also apparent that the insulin sensitizing effect in response to our 3-month mild exercise training program was not tremendously robust, and again, this trend was clearly no longer apparent when subjects abstained from exercise for 3 days after the 3 month training program.

In contrast to many previous studies (21, 22, 57, 67), including some from our laboratory (61, 73), insulin sensitivity in this study was not improved after a single session of exercise (i.e., after the first exercise training session). This finding is also somewhat in contrast to findings from PROJECT #2 of my dissertation in which a single exercise session did enhance insulin sensitivity the next day in a cohort of sedentary overweight adults. An explanation for this discrepancy may be very similar to our rationale for the lack of robust adaptations to the exercise training program; it is likely that the absence of an insulin sensitizing effect of a single session of exercise in the present study may have been a consequence of our rather low exercise stimulus. Therefore, this suggests that the exercise “dose” used in this study may have been below some threshold required to enhance insulin action, even in people who do not exercise regularly. Findings from this study, together with findings from one of our very recent studies (61) may help us better identify the magnitude of an exercise “dose” necessary to induce a persistent improvement in insulin action - at least sustained into the next day. We very recently reported that expending 350 kcals during a single 70 minute session of exercise at 50% VO_2 max improved insulin sensitivity the next morning in a very similar obese cohort as in the present study (61). However, in the current study we found that 40 minutes of exercise at 60%HRmax (i.e., equivalent to ~50% VO_2 max and energy expenditure <200kcal) was not sufficient to improve measures of insulin sensitivity the next day. Obviously, these findings suggest that a minimum “dose” of mild intensity exercise (i.e., ~50% VO_2 max) is somewhere between 40 to 70 min of exercise. It may be quite logical to conclude that the amount of energy expended during the exercise session would be a key element determining the “threshold” for enhancing insulin action, but we cannot rule out the possibility that the duration of exercise may be an important factor, independently of the energy expended. This is based on our earlier finding that expending 350 kcals while exercising at 50% VO_2 max (i.e., ~70 min of exercise) significantly enhanced insulin sensitivity the next morning, but expending 350kcal

while exercising at 65% VO_2max (i.e., ~50 min of exercise) did not (61), suggesting that the duration of exercise may influence this effect. Although perhaps somewhat counter-intuitive, these findings are consistent with other studies suggesting that lower intensity but longer duration exercise may be better at improving insulin sensitivity and blood glucose control in individuals with type 2 diabetes (6, 54).

There has been considerable debate about the role of oxidative capacity on the regulation of insulin resistance (64, 73, 75, 83). In particular, much of this debate has centered around the role that increased fatty acid oxidation may have on reducing the accumulation of intramyocellular lipids known to impair insulin signaling (64, 73, 75, 83). Several reports have suggested that impairments in mitochondrial function and a resultant suppression in fatty acid oxidative capacity are important underlying factors for the development of insulin resistance (8, 51, 68). It has been suggested that by increasing oxidative capacity via exercise training, this could relieve the lipid-induced insulin resistance commonly found in obesity (24, 30, 69). In contrast, fatty acid oxidation is often not found to be low in adults with a moderate degree of obesity (BMI: 30-40kg/m²) (7, 38, 43), and a high capacity to oxidize fatty acids does not necessarily protect against insulin resistance (55, 59, 73). In support of these latter findings, we found that although fatty acid oxidation was augmented by the exercise training program, this was not accompanied by a reduction in intramyocellular lipid concentration, and did not coincide with enhanced insulin action measured the day after exercise. Our data also indicate that mild exercise training without weight loss did not suppress lipolysis or fatty acid mobilization, which is in agreement with previous reports showing that exercise training does not reduce systemic fatty acid availability in weight-stable obese adults (37, 39). Therefore, although the contribution of a low oxidative capacity on muscle lipid accumulation and insulin sensitivity remains to be resolved, exercise training (in the absence of weight loss) does not appear to relieve the persistent physiologic stress of excessive systemic fatty acid overabundance in obese adults.

Systemic and tissue-specific inflammatory stress are considered key factors underlying the increased disease risk in obesity (34, 62, 76), and may play particularly important roles in the development of insulin resistance (62, 76). However, whether changes in systemic, adipose tissue, and/or skeletal muscle inflammation contribute to the exercise-induced reduction in

cardiometabolic disease risk has not been resolved. Adipose tissue expansion and the resultant elevated fatty acid availability that occur with weight gain and the development of obesity are primary contributors to pro-inflammatory macrophage accumulation within adipose tissue (49, 86) and release of pro-inflammatory cytokines into the systemic circulation. In turn, weight loss reverses this macrophage infiltration in adipose tissue (49) as well as markedly suppresses the abundance of systemic pro-inflammatory cytokines (14, 15, 18). In animal models, both acute exercise (63) and exercise training have been reported to reduce pro-inflammatory macrophage infiltration in adipose tissue and lower cytokine expression (47). However, studies in human subjects have reported that neither acute exercise (19, 56, 77) nor exercise training reduced concentration of pro-inflammatory cytokines in obese weight-stable adults (3, 18). Our data support findings from Christiansen, et al, (18) in that neither markers of inflammation in the systemic circulation (i.e., IL-6, TNF- α , and MCP-1) nor adipose tissue (i.e., TNF- α , MCP-1, and VEGF) were reduced by the end of the 3-month exercise program. These findings suggest that in absence of weight loss, low-intensity exercise training, per se, may not affect adipose tissue or systemic inflammation. The influence of exercise on inflammatory pathway activation in skeletal muscle is also complex. A previous study from our lab clearly demonstrated that weight loss (without exercise) suppressed markers of inflammatory signaling in skeletal muscle, yet when exercise training was added to the weight loss program, skeletal muscle inflammation was not reduced any further (73). Therefore, findings from our present study indicating that mild exercise training did not influence skeletal muscle pro-inflammatory stress including NF κ B, I κ B α , and pJNK, are in line with our previous work (73). Much like our conclusions about systemic and adipose tissue inflammation, these findings suggesting that in the absence of weight loss, exercise training (at least rather mild exercise training) may not affect some important inflammatory pathways in skeletal muscle.

We did find that 3-months of mild exercise training without weight loss improved blood lipid profile of our obese participants. Despite the accelerated interest and development of pharmacological treatments for hypercholesterolemia (5, 46), exercise remains a mainstay in the therapeutic approach for improving blood lipid profile. Because higher cardiorespiratory fitness (e.g., VO₂max) is associated with lower cardiovascular disease risk (52) and higher intensity exercise training is often required to induce robust improvements in VO₂max (26), vigorous

exercise is often recommend for improving cardiovascular disease risk factors, like lowering blood lipids. However, our mild intensity exercise program lowered plasma LDL cholesterol, which resulted in a reduction in total cholesterol, without improving VO₂max. How our exercise program lowered plasma LDL-C is not clear. Because triglyceride rich very low-density lipoprotein (VLDL) is a precursor for LDL, a reduction in VLDL could lower LDL. Although we did not measure VLDL and we did not see changes in plasma triglyceride concentration, previous reports have indicated that exercise training can lower plasma VLDL concentration (79, 81). Regardless of the mechanism(s) responsible for lowering LDL-C and Total-C, these changes represent clinically relevant adaptations in response to rather mild exercise, even without weight loss. More specifically we found an 11% reduction in Total-C, which associated with a reduction in relative risk for all-cause mortality by as much as 13% (32, 33). We also found that LDL-C was reduced by ~21 mg/dl, which is well above estimates suggesting that an 11 mg/dl reduction in LDL-C translates to a 9.2% reduction in relative risk for all-cause mortality (31).

The overarching goal of this study was to determine the progressive adaptations associated with adopting a mild exercise training program in weight-stable obese adults. We found that a single 40 minute session of low-intensity exercise was not sufficient to improve insulin sensitivity or reduce other cardiometabolic disease risk factors. However, improvements in insulin sensitivity were apparent after 2 weeks of mild exercise training, but this improvement was very short-lived; measurable improvement in the few hours after exercise, but did not extend into the next day. Importantly, even a relatively short-lived enhancement of insulin action (for the few hours after exercise) may still have profound long-term metabolic health benefits, but to truly reap these metabolic benefits the exercise would need to be performed very regularly (i.e., daily). We also found improvements in blood lipid profile after 3 months of exercise training, even in the absence of weight loss. Although the mechanisms responsible for improvements in cardiometabolic health in response to a mild exercise intervention are not completely understood, these effects may represent a compilation of relatively subtle adaptations in multiple tissues, such as skeletal muscle, liver, and adipose tissue.

Table 5-1. Participant characteristics before and after the 3-month exercise training program

| | Before | After |
|-----------------------------|-------------|-------------|
| Sex (M/F) | (3/9) | – |
| Age (y) | 29.5 ± 2.5 | – |
| Body weight (kg) | 108.2 ± 4.3 | 108.7 ± 4.7 |
| BMI (kg/m ²) | 37.6 ± 1.4 | 38.1 ± 1.5 |
| Body fat (%) | 47.5 ± 2.2 | 47.7 ± 2.5 |
| Fat mass (kg) | 51.6 ± 3.4 | 52.0 ± 3.8 |
| Fat free mass (kg) | 56.6 ± 3.0 | 56.7 ± 3.3 |
| VO ₂ max (L/min) | 1.9 ± 0.1 | 1.9 ± 0.2 |

Values are expressed mean ± SE. BMI, Body mass index; VO₂max, maximal oxygen consumption.

Table 5-2. Skeletal muscle inflammatory proteins

| | PRE | 1EX | 2WKS | 3M | 3d after EX |
|-----------|-----------|-----------|-----------|-----------|-------------|
| | (n=9) | (n=9) | (n=9) | (n=9) | (n=8) |
| NFκB (AU) | 494 ± 60 | 664 ± 106 | 475 ± 73 | 418 ± 132 | 526 ± 63 |
| IκBα (AU) | 529 ± 120 | 561 ± 142 | 502 ± 124 | 617 ± 227 | 731 ± 72 |
| pJNK (AU) | 55 ± 13 | 81 ± 16 | 51 ± 12 | 87 ± 25 | 64 ± 17 |

Values are expressed mean ± SE. PRE: before starting the exercise program; 1EX: the day after the first exercise training session; 2WKS: the day after the 12th exercise session (2 weeks of the exercise program); 3M: the day after the last exercise session at 3 months of the program; 3d after EX: exactly 3 days after the last exercise session after 3 months of the exercise program. NFκB, Nuclear Factor Kappa-B; IκBα, inhibitor of NFκB; pJNK, phosphorylated c-jun N-terminal kinase.

Table 5-3. Basal pAkt^{s473} and pIRS-1^{s312} abundance

| | PRE | 1EX | 2WKS | 3M | 3d after EX |
|------------------|-----------|-----------|-----------|-----------|-------------|
| | (n=9) | (n=9) | (n=9) | (n=9) | (n=8) |
| pAkt s473 (AU) | 6.1 ± 0.7 | 6.1 ± 0.5 | 5.6 ± 0.5 | 7.3 ± 0.8 | 7.5 ± 0.7 |
| pIRS-1 s312 (AU) | 1.3 ± 0.0 | 1.3 ± 0.0 | 1.3 ± 0.0 | 1.3 ± 0.0 | 1.3 ± 0.0 |

Values are expressed mean ± SE. PRE: before starting the exercise program; 1EX: the day after the first exercise training session; 2WKS: the day after the 12th exercise session (2 weeks of the exercise program); 3M: the day after the last exercise session at 3 months of the program; 3d after EX: exactly 3 days after the last exercise session after 3 months of the exercise program. pAkt s473, phosphorylation of protein kinase-B serine residue 473; pIRS-1 s312, phosphorylation of insulin receptor substrate-1 serine residue 312.

Table 5-4. Enzymes involved in IMTG synthesis

| | PRE | 1EX | 2WKS | 3M | 3d after EX |
|------------|-----------|-----------|------------|------------|-------------|
| | (n=9) | (n=9) | (n=9) | (n=9) | (n=8) |
| GPAT (AU) | 6.1 ± 0.7 | 6.1 ± 0.5 | 5.6 ± 0.5 | 7.3 ± 0.8 | 7.5 ± 0.7 |
| DGAT1 (AU) | 9.3 ± 1.1 | 9.4 ± 0.6 | 11.1 ± 0.6 | 10.0 ± 0.5 | 9.3 ± 1.0 |
| DGAT2 (AU) | 2.2 ± 0.1 | 2.2 ± 0.1 | 2.3 ± 0.1 | 2.3 ± 0.1 | 2.3 ± 0.1 |

Values are expressed mean ± SE. PRE: before starting the exercise program; 1EX: the day after the first exercise training session; 2WKS: the day after the 12th exercise session (2 weeks of the exercise program); 3M: the day after the last exercise session at 3 months of the program; 3d after EX: exactly 3 days after the last exercise session after 3 months of the exercise program. GPAT, glycerol-3-phosphate; DGAT1 diglyceride acyltransferase 1; DGAT2 diglyceride acyltransferase 2.

Table 5-5. Glycerol Ra and re-esterification

| | PRE | 1EX | 2WKS | 3M | 3d after EX |
|--|--------------|--------------|--------------|--------------|--------------|
| | (n=9) | (n=9) | (n=9) | (n=9) | (n=8) |
| Glycerol Ra ($\mu\text{mol}/\text{min}$) | 340 ± 49 | 302 ± 33 | 354 ± 49 | 361 ± 47 | 323 ± 37 |
| Glycerol Re-esterification (%) | $49 \pm 4\%$ | $50 \pm 3\%$ | $49 \pm 3\%$ | $52 \pm 6\%$ | $48 \pm 4\%$ |

Values are expressed mean \pm SE. PRE: before starting the exercise program; 1EX: the day after the first exercise training session; 2WKS: the day after the 12th exercise session (2 weeks of the exercise program); 3M: the day after the last exercise session at 3 months of the program; 3d after EX: exactly 3 days after the last exercise session after 3 months of the exercise program.

Table 5-6. Plasma substrate, hormone, and lipid concentrations

| | PRE | 1EX | 2WKS | 3M | 3d after EX |
|--------------------|--------------|--------------|---------------------|---------------------|---------------------|
| | (n=10) | (n=10) | (n=10) | (n=9) | (n=8) |
| Glucose mmol/L | 4.8 ± 0.1 | 4.6 ± 0.1 | 4.5 ± 0.2 | 4.6 ± 0.1 | 4.6 ± 0.1 |
| Insulin uU/mL | 27.1 ± 1.4 | 29.4 ± 1.5 | 29.4 ± 1.3 | 27.1 ± 2.3 | 26.2 ± 2.8 |
| Triglyceride mg/dL | 110.9 ± 20.9 | 115.8 ± 19.3 | 116.8 ± 24.6 | 122.3 ± 28.4 | 114.8 ± 22.1 |
| Total-C mg/dL | 184.0 ± 10.5 | 174.4 ± 9.6 | 176.2 ± 8.7 | 174.8 ± 8.3 | 163.1 ± 6.6* |
| LDL-C mg/dL | 152.3 ± 11.0 | 142.7 ± 10.0 | 144.3 ± 10.0 | 142.1 ± 11.2 | 131.3 ± 9.1* |
| HDL-C mg/dL | 31.7 ± 2.8 | 31.7 ± 2.9 | 31.9 ± 2.7 | 32.5 ± 3.6 | 31.5 ± 3.9 |
| NEFA μmol/mL | 0.39 ± 0.03 | 0.36 ± 0.03 | 0.48 ± 0.04* | 0.50 ± 0.05* | 0.38 ± 0.04 |

Values are expressed mean ± SE. PRE: before starting the exercise program; 1EX: the day after the first exercise training session; 2WKS: the day after the 12th exercise session (2 weeks of the exercise program); 3M: the day after the last exercise session at 3 months of the program; 3d after EX: exactly 3 days after the last exercise session after 3 months of the exercise program.

* Significantly different than PRE, P < 0.05. Total-C, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; NEFA, non-esterified fatty acid.

Table 5-7. Adipose inflammatory proteins

| | PRE | 1EX | 2WKS | 3M | 3d after EX |
|-------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| | (n=9) | (n=9) | (n=9) | (n=9) | (n=8) |
| TNF- α (pg/ μ g protein) | 0.06 \pm 0.02 | 0.05 \pm 0.01 | 0.09 \pm 0.05 | 0.05 \pm 0.01 | 0.05 \pm 0.01 |
| MCP-1 (pg/ μ g protein) | 1.6 \pm 0.4 | 1.2 \pm 0.2 | 1.5 \pm 0.2 | 1.3 \pm 0.1 | 1.7 \pm 0.4 |
| VEGF (pg/ μ g protein) | 7.6 \pm 1.3 | 7.3 \pm 1.4 | 6.0 \pm 0.7 | 5.7 \pm 0.8 | 7.7 \pm 1.5 |

Values are expressed mean \pm SE. PRE: before starting the exercise program; 1EX: the day after the first exercise training session; 2WKS: the day after the 12th exercise session (2 weeks of the exercise program); 3M: the day after the last exercise session at 3 months of the program; 3d after EX: exactly 3 days after the last exercise session after 3 months of the exercise program. TNF- α , tumor necrosis factor-alpha; MCP-1, monocyte chemoattractant protein-1, VEGF, vascular endothelial growth factor.

Table 5-8. Plasma inflammatory proteins

| | PRE (n=9) | 1EX (n=9) | 2WKS (n=9) | 3M (n=9) | 3d after EX (n=8) |
|-----------------------|--------------|--------------|---------------|--------------|----------------------|
| IL-6 (pg/mL) | 5.1 ± 1.6 | 4.7 ± 2.2 | 4.6 ± 2.1 | 5.0 ± 1.7 | 4.3 ± 1.9 |
| TNF- α (pg/mL) | 6.5 ± 0.8 | 6.7 ± 1.0 | 6.8 ± 0.8 | 6.9 ± 1.0 | 5.9 ± 0.8 |
| MCP-1 (pg/mL) | 202.4 ± 27.3 | 178.1 ± 23.0 | 187.8 ± 21.1 | 187.3 ± 21.5 | 163.0 ± 11.5 |

Values are expressed mean \pm SE. PRE: before starting the exercise program; 1EX: the day after the first exercise training session; 2WKS: the day after the 12th exercise session (2 weeks of the exercise program); 3M: the day after the last exercise session at 3 months of the program; 3d after EX: exactly 3 days after the last exercise session after 3 months of the exercise program. IL-6, Interleukin-6; TNF- α , tumor necrosis factor-alpha; MCP-1, monocyte chemoattractant protein-1.

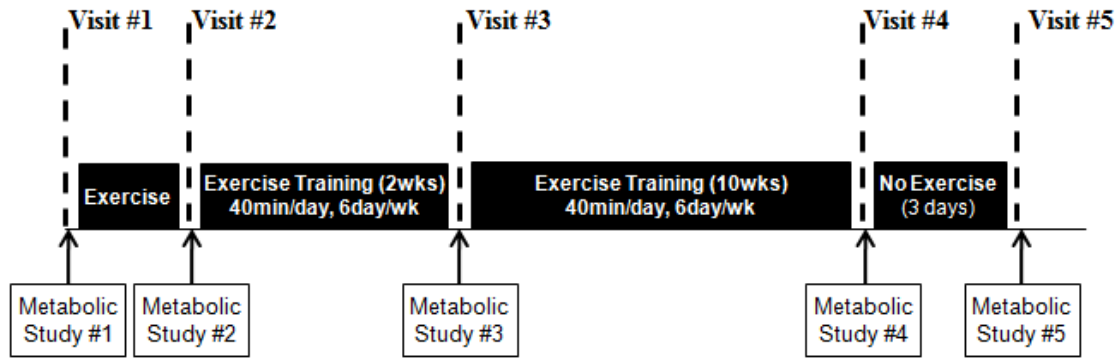


Figure 5-1. Timeline of metabolic studies throughout the 12-14 week participation period.

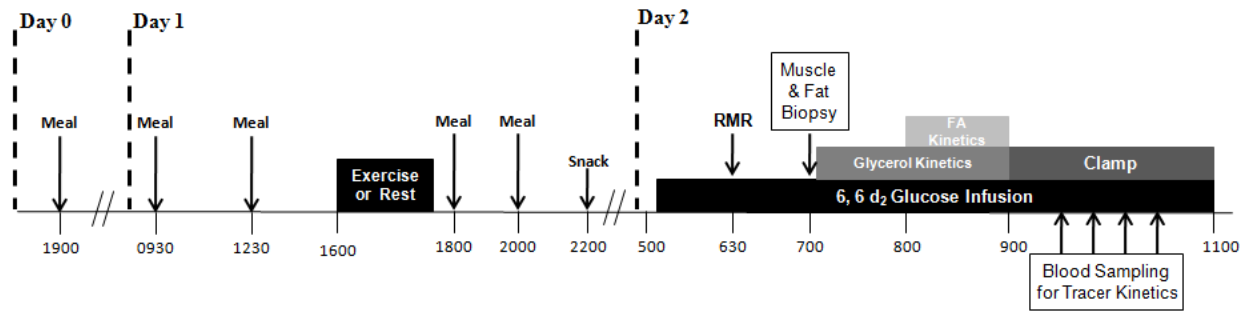


Figure 5-2. Timeline of metabolic tests during each visit. RMR, resting metabolic rate.

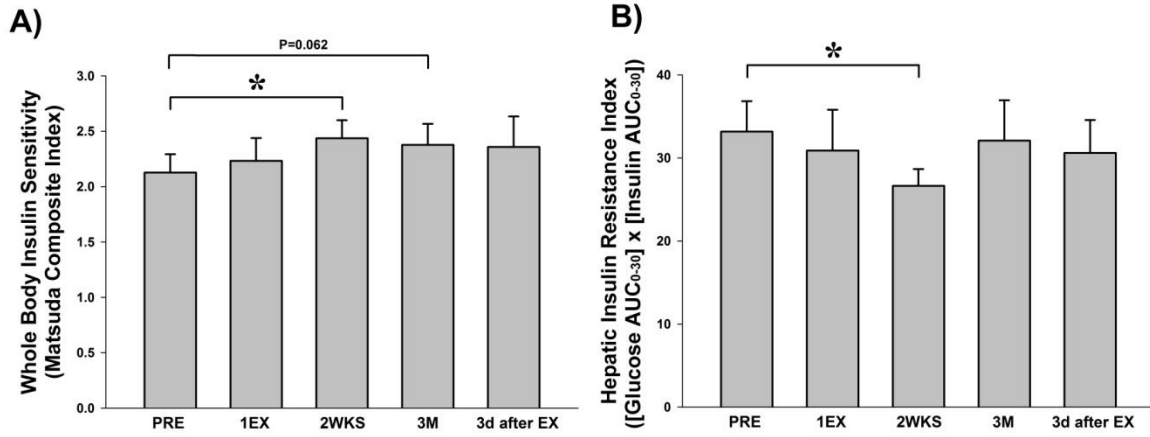


Figure 5-3. (A) Whole Body Insulin Sensitivity Index and (B) Hepatic Insulin Resistance Index during the meal tolerance test before beginning the exercise program (“PRE”), following their first exercise training session (“1EX”), after exactly two weeks of training (“2WKS”), after three months of training (“3M”), and again exactly 3 days after their last session of the 3-month exercise training program (“3d after EX”). *Significantly different than PRE, $P < 0.05$.

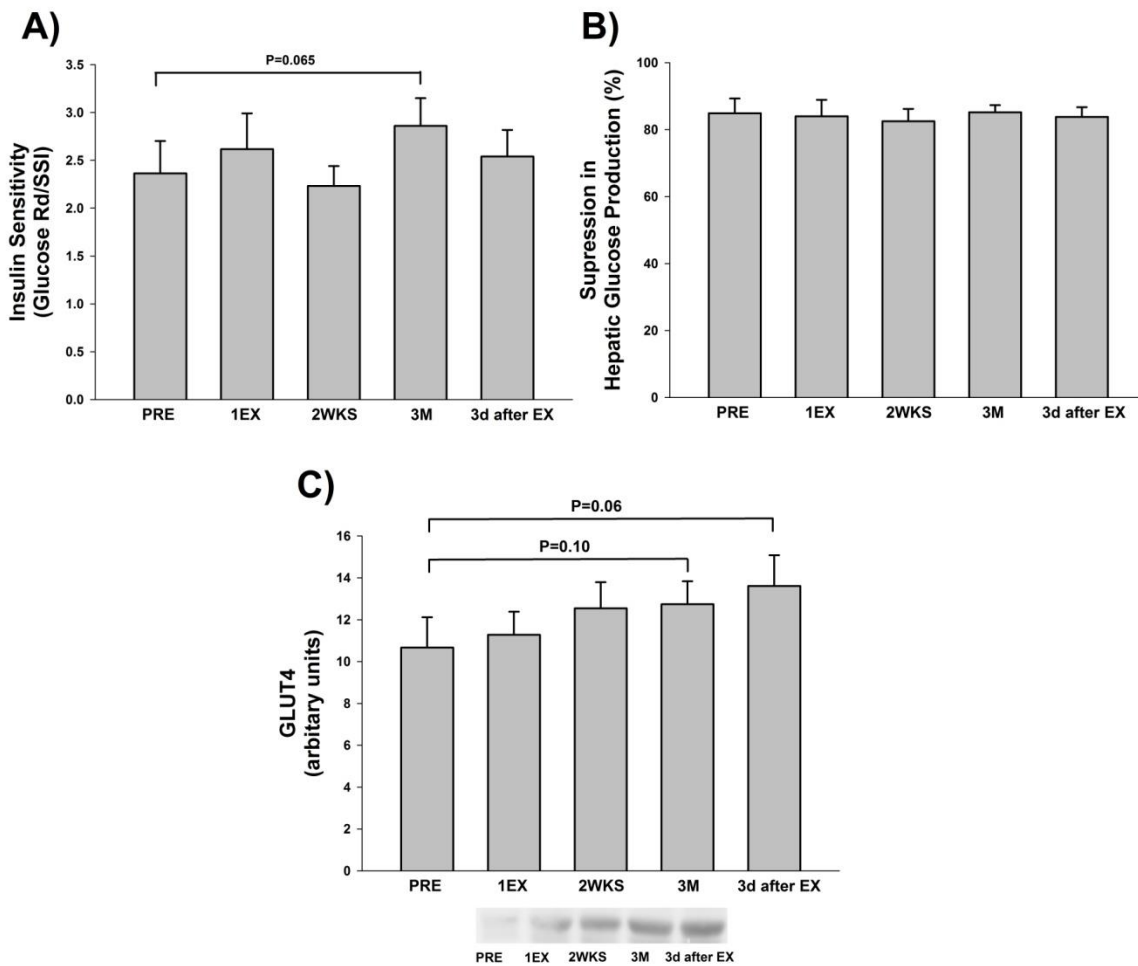


Figure 5-4. (A) Insulin Sensitivity and (B) Suppression of Hepatic Glucose Production during the hyperinsulinemic-euglycemic clamp, and (C) Skeletal muscle GLUT4 abundance before beginning the exercise program (“PRE”), following their first exercise training session (“1EX”), after exactly two weeks of training (“2WKS”), after three months of training (“3M”), and again exactly 3 days after their last session of the 3-month exercise training program (“3d after EX”).

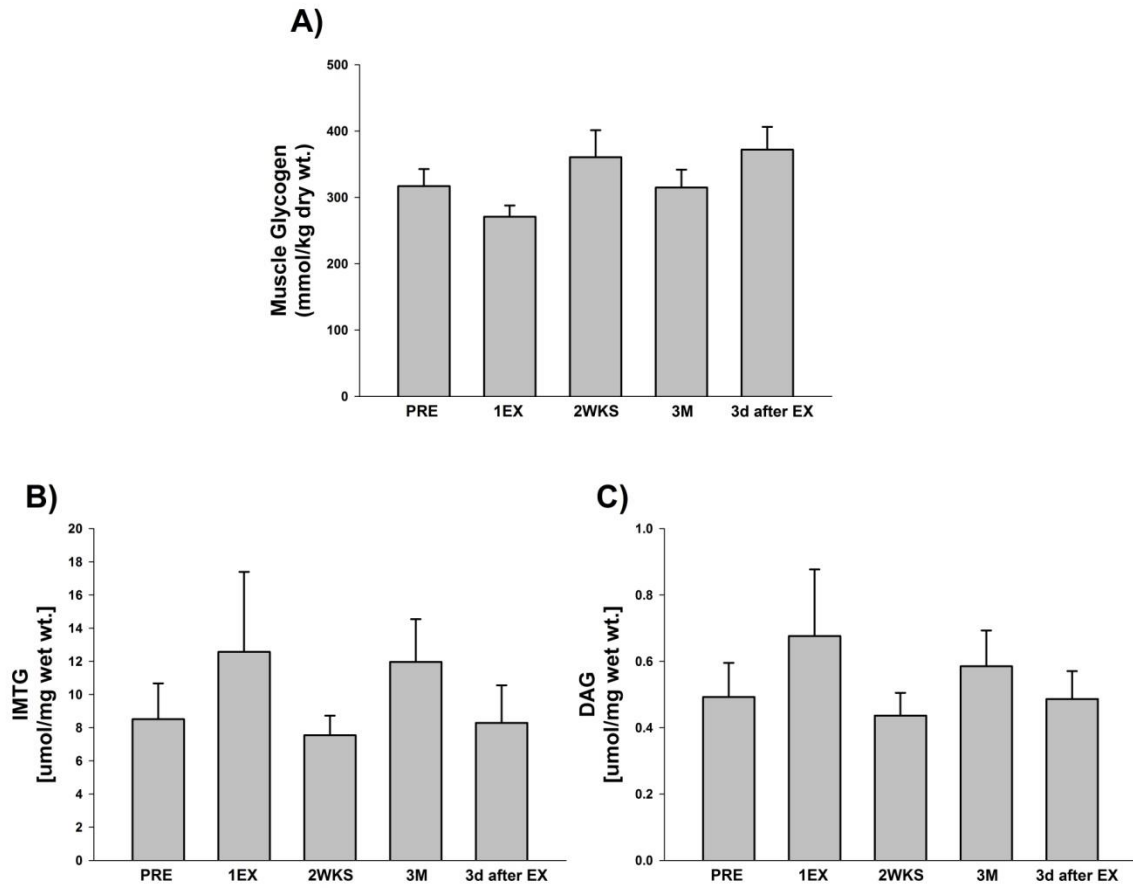


Figure 5-5. Skeletal muscle (A) Glycogen, (B) IMTG, and (C) DAG concentration before beginning the exercise program (“PRE”), following their first exercise training session (“1EX”), after exactly two weeks of training (“2WKS”), after three months of training (“3M”), and again exactly 3 days after their last session of the 3-month exercise training program (“3d after EX”).

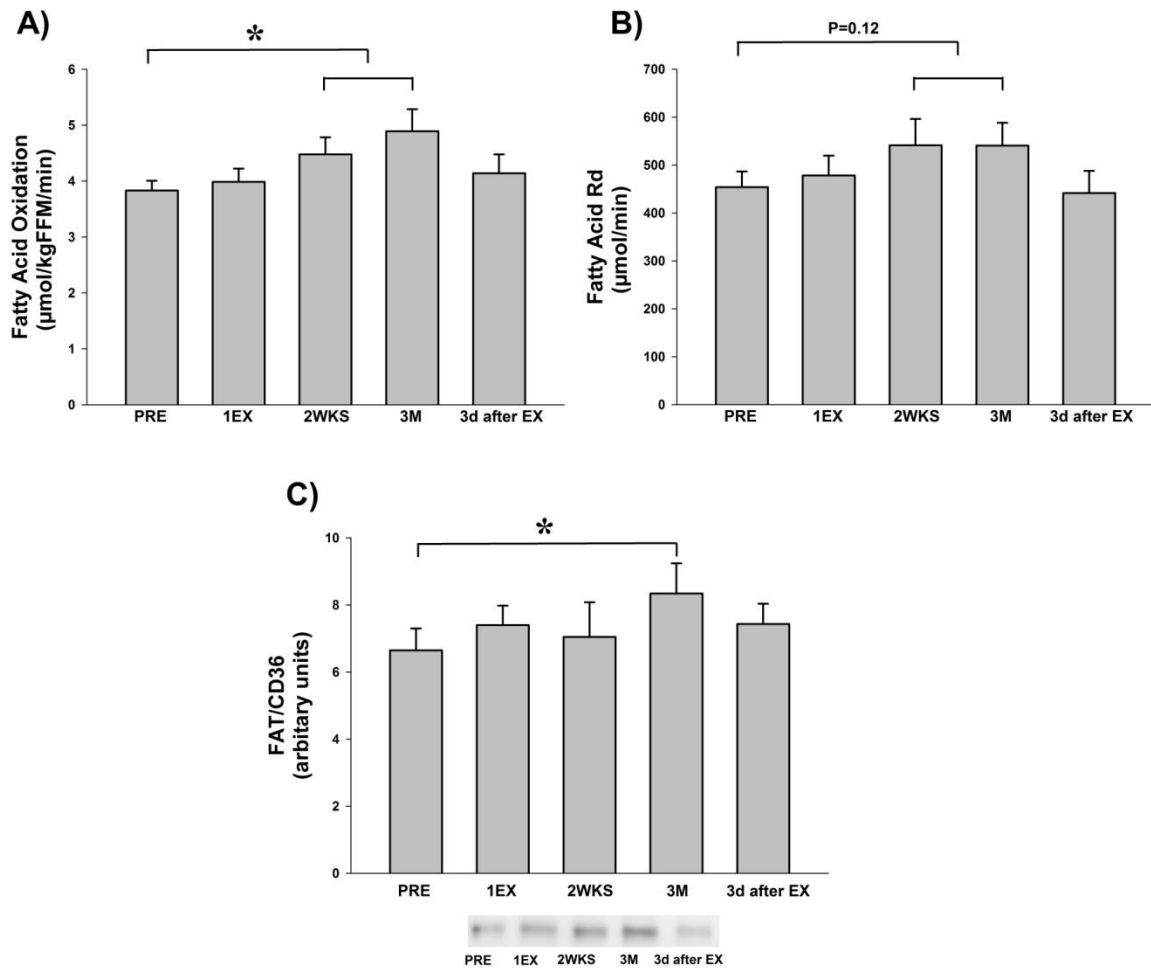


Figure 5-6. (A) Whole body resting fatty acid oxidation, (B) Fatty acid Rd, and (C) Skeletal muscle protein abundance FAT/CD36 before beginning the exercise program ("PRE"), following their very first exercise training session ("1EX"), after exactly two weeks of training ("2WKS"), after three months of training ("3M"), and again exactly 3 days after their last session of the 3-month exercise training program ("3d after EX"). *Significantly different than PRE, $P < 0.05$.

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CHAPTER 6

OVERALL DISCUSSION

A physically active lifestyle is clearly linked with enhanced cardiometabolic health, yet it is very surprising that there are still many unresolved questions about how physical activity/exercise improves important health outcomes. For example, although exercise does not need to be particularly vigorous in order to reduce cardiometabolic disease risk, it remains unclear how relatively mild exercise induces important health benefits, even when the exercise stimulus is below the level often found to be required to induce adaptations classically associated with improved $VO_2\text{max}$. The physiological adaptations stemming from a moderate increase in habitual physical activity behavior and the time course for these adaptations that occur in the early stages of adopting a more physically active lifestyle have not been clearly characterized. Additionally, beneficial metabolic responses in the few to several hours after each exercise session suggests that the transient acute response to exercise might be even more important than adaptations accrued from weeks, months or even years of exercise. However, a clear dissociation between the contributions of transient acute responses to exercise and the more chronic adaptations accrued from a habitual exercise routine on the exercise-induced reduction of cardiometabolic risk factors has not been fully discerned. Collectively my dissertation projects were designed to distinguish between the health benefits derived from acute exercise compared to habitual physical activity/exercise in overweight and obese adults, as well as examined the time course of some key metabolic adaptations that occur as a consequence of an increase in habitual physical activity.

Together the three projects of my dissertation enhanced our understanding of health benefits and underlying mechanisms resulting from acute and habitual physical activity/exercise. Important findings from my dissertation studies include: 1) in a large population study, I established that the degree of physical activity was significantly associated with lower insulin resistance, but $VO_2\text{max}$ was not (PROJECT #1), 2) affirmed that key health benefits can be derived from exercise

independently from of any improvement in $VO_2\text{max}$ (PROJECT #1 and PROJECT #3), 3) demonstrated that reductions in systemic pro-inflammatory stress after a single session of exercise may contribute to the exercise-induced improvements in insulin sensitivity (PROJECT #2), 4) discovered that a single session of exercise at 50% $VO_2\text{max}$ for 40 min may be below a “threshold” for exercise stimulus required to induce a persistent improvement in insulin sensitivity into the next day in obese adults (PROJECT #3), and 5) 3 months of a low-intensity exercise training without weight loss did not alter markers of pro-inflammatory stress or induce persistent improvements in insulin sensitivity among overweight and obese adults. In the discussion that follows, I will focus primarily on the collective and integrative implications of these findings.

A key tenet of my dissertation centered on the important health benefits stemming from a single session of exercise. Given the well-established impact that acute exercise has on key health outcomes, (like insulin resistance) and that many of the health benefits of exercise training “wear off” after even a few days without exercise, it seems logical to develop exercise prescriptions that focus on optimizing the acute responses to EACH exercise session. However, practical issues like “how much” of an exercise stimulus is required for improvement and does this exercise requirement change as the individual adapts to a routine exercise regimen, remain unresolved. In PROJECT #2, I found that a single session of moderate-intensity exercise (~60% $VO_2\text{max}$ for 1 h) was sufficient to improve insulin sensitivity the next day in overweight/obese sedentary subjects. In contrast, in PROJECT #3, when a similar cohort of sedentary obese subjects were exposed to a lower exercise stimulus (50% $VO_2\text{max}$ for 40 min) insulin sensitivity was not improved the next day. It may be important to acknowledge that the methods used to assess insulin sensitivity the day after exercise were different in these two studies. But the hyperinsulinemic-euglycemic clamp was used in PROJECT #3 (i.e., the “gold standard” for assessing insulin sensitivity), and this relatively sensitive method still did not detect improvement in insulin action the day after the mild exercise session. The findings here suggest that the exercise “dose” of about 40 min of exercise at 50% $VO_2\text{max}$ used in PROJECT #3 may be below a minimum threshold necessary to enhance insulin sensitivity into the next day in this population of obese sedentary adults. In another very recent study from our lab (1), we found that a single 70 minute session of exercise at 50% $VO_2\text{max}$ improved insulin sensitivity the next morning, again, using the clamp procedure, in a very similar obese cohort as in the present study. Together these findings suggest

that a minimum “dose” of mild intensity exercise (i.e., ~50% VO_2max) is somewhere between 40 to 70 min of exercise. It is certainly tempting to propose follow-up exercise “dose-response” studies in an attempt to more precisely identify the dose of exercise necessary to induce a persistent improvement in insulin sensitivity (at least into the next day). However, because multiple components contribute to defining an exercise “dose” (e.g., intensity, duration, mode, energy expenditure), and other complexities such as meal timing and content, as well as inter-individual variability will greatly complicate matters, trying to achieve high precision would be very challenging.

Unlike the insulin sensitizing effects of acute exercise in overweight subjects who do not normally exercise, in PROJECT #2 I also found that a single session of moderate exercise did not increase insulin sensitivity the next day in overweight adults who already exercise regularly. The underlying reason for this is not clear, but may be related to less muscle glycogen used during exercise after training, which in turn may attenuate the exercise-induced enhancement of insulin sensitivity. Other training adaptations (e.g., changes in blood flow regulation to the active muscle, increased mitochondrial density) that result in a lower metabolic stress during the exercise session may also contribute to this phenomenon. From a practical perspective, this quite simply suggests that as a person advances in an exercise program, the exercise stimulus may need to increase progressively in order to reap some/much/all of the health benefits of the acute exercise session. This has important clinical implications, and it may be beneficial for exercise/lifestyle prescriptions to incorporate a recommendation to progressively increase exercise intensity.

Although the improvement in VO_2max that often accompanies long-term moderate-to-vigorous exercise training is commonly credited for improvements in health, it is not clear that the physiologic adaptations in skeletal muscle (i.e., increased mitochondrial and capillary density) contributing to the improvement in VO_2max are necessary for the reduction in disease risk factors often found with exercise training. In PROJECT#1, I used what is arguably the most comprehensive health assessment database on the U.S. population, and found average daily minutes of moderate-to-vigorous physical activity was significantly associated with lower insulin resistance, but VO_2max was not. Although probing large data-sets, like NHANES, to address important questions and explore compelling relationships is interesting and impactful, there are

obviously several limitations associated with analysis of these large data sets (e.g., heterogeneous population, no control of the subjects activity and diet before testing, the outcome measures are greatly limited). My findings in PROJECT#1 provided the impetus and foundation for PROJECT #2, in which I recruited a homogeneous population of overweight-to-mildly obese adults to be better able to strictly control their diet and physical activity, and this approach also enabled me to assess their cardiometabolic risk factors more comprehensively. In this study, I found that regular exercisers did exhibit higher insulin sensitivity compared with a well-matched cohort of non-exercisers, even when the exerciser abstained from their regular exercise routine for 3 days, which was in contrast with my original hypothesis. Because our group of regular exercisers in PROJECT #2 also had higher VO₂max than the non-exercisers, I could not distinguish between the metabolic benefits due to the elevated VO₂max compared with other adaptations stemming from exercising regularly. Importantly, in PROJECT #3 significant improvements in both insulin sensitivity and blood cholesterol were found with 3-months of a mild-intensity exercise program without improving VO₂max. Together these studies support our working hypotheses that exercise induced improvements in health may be largely independent of specific physiologic adaptations responsible for improved VO₂max.

The physiological consequences of obesity that lead to reduced cardiometabolic health are complex. However, available evidence suggests that excessive abundance of fatty acids, accumulation of deleterious lipid species (i.e., DAG and ceramide) and increased pro-inflammatory stress within skeletal muscle may be mediators of reduced health and in particular insulin sensitivity among obese adults. Therefore, a working hypothesis of our laboratory is that exercise may improve insulin sensitivity in obese adults in part through altering the metabolic fate of fatty acids that enter the muscle cell, thereby reducing pro-inflammatory stress within skeletal muscle. This hypothesis is largely based on previous findings from our laboratory by Dr. Schenk and Dr. Newsom who examined the effects of acute exercise on skeletal muscle lipid partitioning and pro-inflammatory stress. For example, Dr. Schenk found that compared with remaining sedentary, a single session of vigorous exercise protected against lipid induced insulin resistance in lean adults. Importantly this was accompanied by an increase in accumulation of neutral lipid stores (IMTG) and reduced accumulation of negative lipid species (i.e., DAG and ceramide) as well as skeletal muscle pro-inflammatory stress. Additionally, Dr. Newsom found that a single

session of low-intensity exercise increased insulin sensitivity in obese adults with only modest changes in muscle lipid accumulation and no change in pro-inflammatory stress. While these earlier projects focused exclusively on the important metabolic effects of acute exercise, the projects of my dissertation expanded on these findings by examining the effects of cumulative sessions of exercise and exercise training (in addition to acute exercise responses) on insulin sensitivity, skeletal muscle lipid-partitioning and pro-inflammatory stress. My projects also extend on previous work in our lab by peering into the potential effects of exercise on adipose tissue inflammation and cytokine production. Accumulating evidence suggests that adipose tissue and systemic pro-inflammatory stress contribute to insulin resistance among obese adults. However, it is not clear that improvements in insulin sensitivity following exercise training are mediated by reductions in adipose tissue or systemic pro-inflammatory stress especially among weight stable overweight and obese adults. In PROJECT #2, I found that increased insulin sensitivity following a single session of moderate intensity exercise was significantly associated with reductions in pro-inflammatory cytokine IL-1 β . Although we cannot assume a cause-and-effect relationship with these findings, these results support growing evidence indicating that exercise training induced reductions in systemic pro-inflammatory stress may contribute to exercise induced improvements in insulin sensitivity. Conversely in PROJECT #3 neither an acute session of low-intensity exercise nor low-intensity exercise training relieved markers of adipose tissue, systemic, or skeletal muscle pro-inflammatory stress. Additionally we did not see changes in accumulation of IMTG or DAG within skeletal muscle. As noted above, it is possible that the exercise stimulus in PROJECT #3 may not have been sufficient to induce these changes.

A better understanding of the cardiometabolic health benefits associated with acute and habitual exercise training has certainly been gained from the results of these dissertation projects. However, questions still remain. Due to the cross-sectional study design of PROJECT #1 we cannot determine causality between the inverse relationship in habitual physical activity level and insulin resistance. It is possible that the persistent effect of regular exercise training on insulin sensitivity observed in PROJECT #2 was, at least in part, due to the physiologic adaptations associated with enhanced VO₂max. However in PROJECT #3, clinically relevant improvements in indices of insulin sensitivity and blood cholesterol were apparent after just two weeks and three months, respectively of a mild exercise training program that did not result in a significant increase in

VO₂max. Therefore, although significant improvements in key health outcome measures were observed with exercise training in the absence of improved VO₂max, it cannot definitively be determined whether enhanced insulin sensitivity following exercise training is independent of physiologic adaptations leading to increased VO₂max. Finally although reductions in pro-inflammatory cytokine IL-1 β was associated with enhanced insulin sensitivity in PROJECT #2, it is not clear that this relationship is casual, and/or if other inflammatory markers and pathways may be impacted more profoundly by exercise and exercise training.

In summary my dissertation projects support our current understanding that important improvements in metabolic health can be derived from a single session of exercise in overweight/obese adults given a “sufficient” exercise stimulus. These studies also support the notion that exercise induced reductions in pro-inflammatory stress may be a key mediators in exercise induced improvements in insulin sensitivity. Notably these studies assert the importance of regular exercise in combating the physiologic consequences of obesity. Importantly I have shown that persistent improvements in insulin sensitivity and blood cholesterol can be derived from regular exercise in overweight/obese adults. Finally, these studies confirm that clinically meaningful improvements in health can be achieved in the absence of increase in VO₂max. Together, these findings can help inform the development of more effective exercise/lifestyle strategies with the objective of reducing cardiometabolic risk factors in overweight and obese adults.

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APPENDICES

APPENDIX 1

Additional Analyses for PROJECT #1 (Chapter 3):

Multiple linear regression analysis examining the independent associations of physical activity and cardiorespiratory fitness on some key cardiorespiratory disease risk factors

In my dissertation proposal, I indicated that I would examine the independent associations of objectively measured physical activity and cardiorespiratory fitness on insulin sensitivity as well as other measures of metabolic and cardiovascular health (i.e., mean arterial pressure, plasma cholesterol, and c-reactive protein (CRP)). In Chapter 3 of my dissertation, I have provided the manuscript from this study that was very recently accepted for publication (5). In the process of revising this manuscript for publication we were asked to simplify the scope of the project and only focus on the independent associations between physical activity and cardiorespiratory fitness and insulin sensitivity. As a result, findings for the other outcome measures I proposed were not include in the manuscript (and do not appear in Chapter 3 of my dissertation). I have included these data here.

Methods summary

Additional multiple linear regressions were performed with mean arterial pressure (Model 1), total cholesterol (Model 2), and log c-reactive protein (Model 3) as the dependent variable. C-reactive protein was transformed using the log function to correct for the skewed distribution. Minutes of MVPA and VO₂max were the primary predictor variables. Appropriate confounding variables including: adiposity, sex, and age were also included in our complete model.

Results

The cohort of participants used for this analysis was identical to those used in Chapter 3 and participant characteristics were previously presented (Chapter 3, Table 3-1). In our complete models for mean arterial pressure (MAP; Model 1), total cholesterol (Total-C; Model 2), and log c-reactive protein (log CRP; Model 3), that included predicting (cardiorespiratory fitness and

MVPA) confounding variables (body fat percentage, age and sex), neither cardiorespiratory fitness nor MVPA were significantly correlated with MAP, Total-C, or log CRP. Our analysis did not show any evidence of moderation when stratified by sex (data not shown).

Discussion

In this additional analysis we found that neither cardiorespiratory fitness nor MVPA were significantly associated with MAP, Total-C, or CRP. These findings were in line with our observation in PROJECT #2 that blood pressure, blood lipids, and markers of systemic inflammation were similar between sedentary and regular exercising overweight but otherwise healthy (i.e., no signs of hypertension or dyslipidemia) adults. Conversely in PROJECT #3 we found that adapting a mild exercise training program resulted in significant reductions in Total-C driven by a reduction in low-density lipoprotein cholesterol (LDL-C) among previously sedentary but obese adults. Although participants in PROJECT #3 were also otherwise healthy they did show less than “ideal” LDL-C. As previously discussed, reductions in markers of cardiometabolic disease risk following exercise training are typically quite modest in the absence of weight loss (3, 4) but slightly more robust in individuals with additional increased risk due to diagnosed dyslipidemia and/or hypertension (1, 2). Importantly this regression analysis was limited to healthy adults between the ages of 18-45 with no history of diabetes (or taking medication to treat diabetes), cardiovascular or renal disease, stroke or emphysema. Therefore, in line with findings from PROJECT #2 and PROJECT #3 the fact that we did not detect a significant association between either of our predictor (e.g., cardiorespiratory fitness and MVPA) and outcome variables (e.g., MAP, Total-C, and CRP) may be reflective of cohort of healthy participants used in this analysis.

Appendix 1 Table A. Linear regression models predicting mean arterial pressure, cholesterol, and c-reactive protein

| Beta (P-value) | Model 1 (MAP) | Model 2 (Total-C) | Model 3 (log CRP) |
|--|--------------------------|------------------------------|------------------------------|
| Physical Activity (30 min of MVPA ^a) | -1.0395 (0.201) | 4.4493 (0.292) | -0.1549 (0.075) |
| Estimated VO2 Max ^b (ml/kg/min) | -0.1099 (0.142) | -0.2438 (0.363) | -0.0063 (0.499) |
| Body Fat (%) | 0.1952 (0.013) | 0.9230 (0.046) | 0.0892 (<0.001) |
| Age (yrs.) | 0.3465 (<0.001) | 1.2211 (<0.001) | -0.0026 (0.767) |
| Sex Male = 1; Female = 0 | -8.418 (<0.001) | -10.1801 (0.025) | -0.6608 (0.001) |
| Adjusted r ² | 0.238 | 0.140 | 0.257 |

For all models n=402. ^a moderate-to-vigorous physical activity ^b maximal oxygen consumption

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APPENDIX 2

Physical Activity Questionnaire

Subject ID:

Date:

Protocol:

| How often do you participate in the following activities? | Exercise Frequency during the week | | | | | | | | | |
|--|------------------------------------|---------|-------|-------|-------|-------|--------|--------|---------|--------------|
| | None | Minutes | | | | Hours | | | | |
| | | <15 | 15-30 | 30-45 | 45-60 | 1-1.5 | 2 to 3 | 4 to 6 | 7 to 10 | More than 11 |
| Walking | | | | | | | | | | |
| Jogging | | | | | | | | | | |
| Running | | | | | | | | | | |
| Biking | | | | | | | | | | |
| Stationary Bike | | | | | | | | | | |
| Swimming | | | | | | | | | | |
| Tennis, raquetball | | | | | | | | | | |
| Basketball | | | | | | | | | | |
| Dancing | | | | | | | | | | |
| Rock Climbing | | | | | | | | | | |
| Hiking | | | | | | | | | | |
| Gymnastics | | | | | | | | | | |
| Gardening, house work | | | | | | | | | | |
| Skiing, skating | | | | | | | | | | |
| Martial arts | | | | | | | | | | |
| Weight Lifting | | | | | | | | | | |
| Football | | | | | | | | | | |
| Soccer | | | | | | | | | | |
| Aerobics | | | | | | | | | | |
| Rowing | | | | | | | | | | |
| Yoga, pilates | | | | | | | | | | |
| Elliptical | | | | | | | | | | |
| Other: | | | | | | | | | | |
| Please Describe other activity here | | | | | | | | | | |

APPENDIX 3

Skeletal muscle lipid analysis

Skeletal Muscle Diacylglycerol and Triacylglycerol: Purification by Solid Phase Extraction, Thin Layer Chromatography, Gas Chromatography & Mass Spectrometry

A. Preparation of Total Muscle Lipid Extract¹

- 1) Pre-weighed frozen muscle (~30 mg) rapidly homogenized in a ground-glass homogenizer (room temp) containing:
 - a) 0.5 ml chloroform (CHCl_3) + butylhydroxytoluene (BHT)² (10 $\mu\text{g}/\text{ml}$) + internal lipid standards³
 - b) 1.0 ml methanol (MeOH)
 - c) 0.4 ml 0.9% NaCl (saline)
- 2) Homogenate transferred to 16x125 mm extraction tube using a Pasteur pipet; save pipet
- 3) Homogenizer rinsed twice with 1.9 ml volumes of the solvent mix [CHCl_3 + BHT] + MeOH + saline (1:2:0.8); rinses added to extraction tube with saved pipet
- 4) Capped tube vortexed gently 1.5h at room temp → lipids extracted into ~5.7 ml of a single phase solvent mix

B. Isolation of the Lipid Phase

- 1) After room temp incubation, the following added in this sequence (with vortexing):
 - 1st: 1.5 ml [CHCl_3 + BHT]
 - 2nd: 1.5 ml saline

Notes: CHCl_3 addition before saline improves yield

CHCl₃:MeOH:saline ratio changes to 1:1:0.9, forming a two-phase system with lipid-containing CHCl₃ as the lower phase (3.0 ml)

- 2) Tube centrifuged at ~2,300 x g for 10 min at room temp to sharpen interface
- 3) Upper phase + precipitated protein at interface removed by aspiration
- 4) Lipid phase transferred to clean tube and dried in speed-vac w/o heat

Note: avoid excessive drying time; tube in moving rotor warms after solvent evaporates, even under partial vacuum and without heating the chamber

C. Lipid Application to Bulk Silica

- 1) 3.0 ml of 15% ethyl acetate (EtOAc) in hexane added to the lipid residue to dissolve diacylglycerol (DAG), triacylglycerol (TAG) and other lipids of progressively lower polarity; tube vortexed 30 sec

Notes: monoacylglycerol (MAG) is minimally soluble in 15% EtOAc/hexane, and phospholipid (PL) is very poorly soluble

some MAG and PL will remain bound to the glass surface of the tube

- 2) Solution transferred to extraction tube containing 500 mg normal phase silica⁴; tube vortexed and dried

D. Extraction and Removal of Cholesteryl Esters

- 1) 5.0 ml of 0.25% EtOAc/hexane added and tube vortexed 2 min
- 2) Tube centrifuged to compact silica; supernatant containing cholesteryl esters (CE) removed
- 3) Silica rinsed with another 5.0 ml volume of 0.25% EtOAc/hexane as above

E. Extraction of an Enriched [DAG+TAG] Fraction

- 1) Steps in section D again performed, except using 15% EtOAc/hexane to extract mainly DAG and TAG
- 2) Supernatants (10 ml) combined, dried and stored at -20°C

Note: MAG and PL that transfer (see section C) are largely retained in the silica during this [DAG+TAG] extraction

F. Purification of DAG and TAG by Thin Layer Chromatography (TLC)⁵

- 1) Plate Preparation:
 - a) 19-channel, normal phase TLC plates with preadsorbent sample streaking area⁶ heated at 130°C for 30 min in GC oven to remove residual water vapor
 - b) Plates cooled to room temp, then run in MeOH in TLC chamber until solvent front reaches top of plates
 - c) Plates air-dried in fume hood and stored in an air-tight container with desiccant until sample application
- 2) Sample Application:
 - a) [DAG+TAG] residue reconstituted in 35 µl 15% EtOAc/hexane; the entire recoverable volume was streaked in a channel loading area
 - b) Second 35 µl vol added to sample tube, and this streaked in a second channel adjacent to the first
 - c) Mix of the internal standard lipids streaked in separate channels
 - d) Streaks air-dried thoroughly by placing plate atop GC oven (warm surface)
- 3) TLC Run:
 - a) TLC chamber equilibrated with solvent mixture for 1h
solvent mix = hexane + diethyl ether + glacial acetic acid (70:30:1)
 - b) After chamber equilibration, solvent depth adjusted to ~3 mm
 - c) Plates placed in chamber rack, and rack then inserted into chamber to start run
 - d) TLC ended when solvent front reached 1.5 cm from plate tops by removing rack from chamber
 - e) Plates thoroughly air-dried in fume hood, then carefully wrapped in aluminum foil and stored in a freezer bag at -20°C until lipid recovery step

G. Lipid Recovery, Alkaline Methanolysis and GCMSD: done first with TLC channels having lipid standards only → information used for muscle lipid quantification

- 1) Internal Lipid Standard Recovery:
 - a) In a standard channel, fifteen 1 cm-silica segments collected above the loading area; upper 2.5 cm of the loading area (streak zone) also obtained; scraping done with a vacuum-assisted collection device
 - b) Silica samples transferred to 16x100 mm extraction tubes and stored at -20°C

- 2) Alkaline Methanolysis⁷ of Internal Standards:
 - a) 1.0 ml 15% EtOAc/hexane added to silica; tube vortexed 30 sec and centrifuged; 50 µl supernatant transferred to a 12x75 mm tube and dried
 - b) 250 µl 0.6N sodium hydroxide (NaOH) in MeOH added to dried residue; tube vortexed and incubated at room temp for 30 min

Note: during this incubation fatty acids convert from glyceryl or cholesteryl esters to methyl esters
 - c) Reaction stopped by addition of 500 µl 1.0M sodium acetate (NaOAc) buffer, pH4.75
 - d) FAMES extracted into 1 ml hexane; tube vortexed after hexane addition and then centrifuged; hexane w/FAMES (upper phase) transferred to clean tube and dried

- 3) Gas Chromatography & Electron-Impact Mass Spectrometry⁸:
 - a) FAME residue dissolved in 50 µl heptane and transferred to analyzer vial insert
 - b) 1 or 2 µl injected into GCMS; selected ions monitored (typically ions 74, 79 and molecular) at retention times for individual FAMES
 - c) Channel location and purity of DAG, TAG and other lipid standards determined

- 4) Muscle DAG and TAG Recovery and Processing:
 - a) Internal std-determined channel sections (~2.5 cm) for muscle DAG or TAG collected as above; same sections pooled from the two channels used per sample
 - b) Alkaline methanolysis performed as above, except the entire DAG sample used; 0.25 ml (from 1 ml) of the TAG sample used
 - c) GCMSD as above

H. Basic Calculations

- 1) FAME ion peak area values corrected for background using assay blanks:
 - a) Blank 1 = w/o muscle, w/ internal stds → for endogenous FA corrections
 - b) Blank 2 = w/o muscle, w/o internal stds → for internal std corrections

- 2) Conversion of ion peak areas to “picomoles injected” for each FA species measured, using individual FAME standard curves
- 3) Conversion of “pmol FA injected” to “nmol lipid-derived FA / sample” by dividing the pmol value by the fractional recovery of the internal std-derived FAME (C15:0ME for DAG, C17:1ME for TAG), and then dividing by 1000 (pmol to nmol)
- 4) Calculation of nmol DAG (or TAG) / mg muscle wet weight = summed nmol DAG (or TAG)-derived fatty acids / 2 (or 3) / mg wet wt
- 5) Calculation of FA profile for DAG or TAG = nmol each FA / sum x 100

Superscript

1. Lipid extraction adapted from:
 - a) Bligh EG and WJ Dyer. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 8: 911-917, 1959. (primary reference)
 - b) Tepper AD and WJ Van Blitterswijk. Ceramide mass analysis by normal-phase high-performance liquid chromatography. *Methods Enzymol* 312: 16-22, 2000. (secondary reference)
2. BHT (Sigma) = 3,5-Di-tert-butyl-4-hydroxytoluene
Acts as an anti-oxidant; protects unsaturated lipids from oxidative rancidification
3. Internal lipid standards added to homogenizer in 0.5 ml CHCl₃:

| | | |
|--|---------------------------|-----------|
| a) Cholesteryl tridecanoate (Nu-Chek) | [C13:0]-CE | 10.0 nmol |
| b) Dipentadecanoin (Nu-Chek) | [C15:0] ₂ -DAG | 10.0 nmol |
| c) Heptadecanoic acid (Nu-Chek) | [C17:0]-NEFA | 20.0 nmol |
| d) Triheptadecenoin (Nu-Chek) | [C17:1] ₃ -TAG | 16.0 nmol |
| e) Mononadecanoin (Nu-Chek) | [C19:0]-MAG | 12.0 nmol |
| f) Dinonadecenoin (Nu-Chek) | [C19:1] ₂ -DAG | 20.0 nmol |
| g) 1,2-Diheneicosanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids) | [C21:0] ₂ -PC | 16.0 nmol |
| h) Tritricosanoin (Nu-Chek) | [C23:0] ₃ -TAG | 2.0 nmol |
4. Silica = Supelclean LC-SI (Supelco)

5. TLC adapted from:
Kaluzny MA, Duncan LA, Merritt MV and DE Eppse. Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J Lipid Res* 26: 135-140, 1985.

6. Whatman Linear-K Preadsorbent TLC plate, 60A pore diameter

7. Alkaline methanolysis adapted from:
Bodenec J, Brichon G, Zwingelstein G and J Portoukalian. Purification of sphingolipid classes by solid-phase extraction with aminopropyl and weak cation exchanger columns. *Methods Enzymol* 312: 101-114, 2000.

8. GCMSD equipment and major method aspects:
 - a) Agilent 6890A gas chromatograph w/autoanalyzer tray and injector tower
 - b) Agilent 5973N mass spectrometer
 - c) Restek FAMEWAX GC column, 30 meter, 0.25 mm ID, 0.25 μm film thickness, crossbonded polyethylene glycol film
 - d) Helium carrier gas, 1.0 ml/min
 - e) Pulsed splitless injection (generally 1 μl)
 - f) Initial oven temp = 130°C, hold 1 min
Ramp = 10°C/min
Final temp = 240°C, hold 10 min

APPENDIX 4

Exercise adherence data

| | 1EX | 2WKS | | 3M | | |
|----------------|-------------------------------------|-------------------|-------------------------------------|-------------------|-------------------------------------|--|
| Subject ID | Exercise Intensity (%HR max) | Compliance | Exercise Intensity (%HR max) | Compliance | Exercise Intensity (%HR max) | Weeks of Training before Visit #4 |
| PA1 | 62.0% | 100.0% | 61.0% | 95.8% | 66.5% | 12 |
| PA2 | 61.0% | 100.0% | 61.1% | 100.0% | 61.9% | 12 |
| PA3 | 62.0% | 91.7% | 63.7% | 77.8% | 62.0% | 12 |
| PA4 | 63.0% | 83.3% | 60.6% | 90.5% | 59.9% | 14 |
| PA8 | 61.0% | 108.3% | 61.0% | 98.5% | 60.5% | 11 |
| PA9 | 66.0% | 100.0% | 61.1% | 93.6% | 60.5% | 13 |
| PA11 | 63.0% | 100.0% | 62.1% | 94.4% | 61.6% | 12 |
| PA12 | 62.0% | 100.0% | 62.5% | 87.8% | 59.8% | 14 |
| PA13 | 62.0% | 108.3% | 61.0% | 86.8% | 60.4% | 13 |
| PA15 | 63.6% | 100.0% | 61.5% | | | |
| Average | 62.6% | 99.2% | 61.5% | 91.7% | 61.5% | 12.56 |
| SD | 1.5% | 7.3% | 1.0% | 6.9% | 2.0% | 1.01 |
| SE | 0.5% | 2.3% | 0.3% | 2.3% | 0.7% | 0.34 |