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The early time course of autophagy in human peripheral blood mononuclear cells following endurance exercise

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EARLY TIME COURSE OF AUTOPHAGY IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS FOLLOWING ENDURANCE EXERCISE

by

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DISSERTATION

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Early Time Course of Autophagy in Human Peripheral Blood Mononuclear Cells Following Endurance Exercise

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ABSTRACT

Exercise disrupts homeostasis and leads to the induction of an important catabolic system called autophagy. Autophagy is a beneficial cell survival process that is induced in periods of starvation. The purposes of this study are to (1) determine the time course of autophagy activation following endurance exercise at 70% of VO_2max in a warm environment and (2) to determine if exercising at 50% of VO_2max induces autophagy in a warm environment. **Methods.** Eight endurance trained subjects (2 females) participated in this study and completed a moderate intensity exercise (MIE) trial for 1h (50% of VO_2max), and a high intensity exercise (HIE) trial for 1h (70% of VO_2max). **Results.** Core temperature and heart rate during HIE was higher during 10-60 and 5-60 minutes, respectively, when compared to the same time during MIE and pre-HIE, $p < 0.01$. IL-6

levels were increased ($p < 0.01$) 0h post-exercise and 1h post-exercise HIE versus pre-exercise. IL-6 was increased following MIE 0h post-exercise, when compared to pre-exercise, $p < 0.01$. Decreases ($p < 0.05$) in plasma insulin were found following HIE at 2h, when compared to pre-exercise. Decreases in plasma insulin were also found at 4h post-exercise following MIE when compared to pre-exercise, $p < 0.05$. HIE increased ($p < 0.05$) autophagy marker LC3-II at 0h , 2h, and 4h post-exercise when compared to pre-exercise. MIE increased LC3-II at 1h post-exercise when compared to pre-exercise. LC3b decreased following MIE at 1h ($p < 0.01$) and was increased at 2h, post-exercise when compared to baseline. HSPA1A was decreased at 1h following HIE, when compared to baseline, $p < 0.01$. HSP70 and LC3-II were moderately and significantly related during MIE, $p < 0.01$. Increased Akt phosphorylation occurred 2h post-MIE when compared to pre-exercise levels, $p \leq 0.01$. **Conclusions.** Our data suggest that autophagy can be stimulated by exercise at both 50% VO_2max and 70% VO_2max. MIE induced phosphorylation of Akt post-exercise and may be activated independent of circulating insulin levels. It is unknown how or if the decreased levels we observed in plasma insulin and increases in IL-6 influence autophagy in PBMCs following exercise.

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SYMBOLS / ABBREVIATIONS

≥: greater than or equal to

>: greater than

≤: less than or equal to

<: less than

±: plus or minus

~: approximately

°C: degrees Celsius

µg: microgram

ml: milliliter

µl: microliter

µmol: micromolar

ANOVA: analysis of variance

BF%: body fat percentage

bpm: beats per minute

cm: centimeters

dH_2O: distilled water

diH_2O: deionized water

ELISA: enzyme-linked immunosorbent assay

FFM: fat free mass

g: gram

H_2O: water

HR: heart rate

HSP70: heat shock protein 70

IL-6: interleukin-6

IU: International insulin unit

kg: kilogram

LC3-II: microtubule-associated protein light chain 3

M: molar

MAP1LC3B: gene symbol microtubule-associated protein light chain 3 (LC3B)

mg: milligram

mM: milimolar

n: number of subjects

NaCl: sodium chloride

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

pg: picogram

R^2: coefficient of determination

r: Pearson coefficient

RPE: rate of perceived exertion

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of the mean

Tris: tris (hydroxymethyl) aminomethane

VO_2max : maximal oxygen consumption

CHAPTER I

INTRODUCTION

The benefits of regular physical activity include improved health outcomes for people with many chronic diseases; reduced stress and depression; and increased emotional well-being, energy level, confidence and satisfaction with social activity (20). Physiologically, the benefits of long term physical activity range from the whole organ to the cellular level and include protection from disease, defense against metabolic disorders, reduced inflammation, increased longevity, and increases in cellular quality control (6, 17, 27, 32, 44). The economic costs of physical inactivity in New Mexico are substantial but difficult to quantify due in part to the high prevalence of heart disease, diabetes, and interrelationships with other modifiable risk factors of chronic disease (20). Physical activity is commonly included in health behavior studies, though questions about the most optimal frequency and intensity of exercise needed to provide health benefits still remain. Exercise elicits cellular disturbances that disrupt homeostasis and disturbances lead to the induction of an important catabolic system called autophagy (18, 42, 45). Autophagy is a beneficial cell survival process that is induced during periods of negative caloric balance such as exercise or starvation, and functions to recycle cellular proteins and organelles to provide an alternative source of energy (35). Failure of the cell to dispose of byproducts is thought to induce formation of protein aggregates that are observed in Alzheimer's disease, diabetes, heart disease, muscle disease, and cancer and are associated with poor health outcomes and decreased longevity (4, 8, 15, 16, 19, 40).

Mammalian cells rely on strict quality control mechanisms that regulate intracellular damage to maintain homeostasis (19). Autophagy encompasses the different

pathways cells use to deliver cargo to a lysosome for degradation. These may include macroautophagy, chaperone-mediated autophagy, and microautophagy and differ by the means through which they deliver cargo (25). Macroautophagy is the process in which portions of the cell's cytoplasm are sequestered within a double membrane vesicle and delivered to the lysosome and will now be referred to as "autophagy" (15). Failure of the cell to dispose of altered proteins is thought to contribute to the formation of inclusion bodies associated with intermediate filaments and ubiquitin (4). Autophagy preserves cell metabolism and function by participating in the creation of energy sources in times of amino acid deficit; it also is the mechanism for removal of such damaged structures and bodies (40, 43). Through its roles in survival and homeostasis, autophagy is important in human pathologies such as neurodegenerative diseases, cancer, lysosomal diseases, and aging (1, 7, 37, 38).

Physical exercise is also known to modulate glucose homeostasis and is a primary preventative and treatment factor in Type II diabetes (47). Glucose homeostasis and autophagy share activation mechanisms (17). In a recent and ground-breaking study published in *Nature*, researchers found that autophagy-deficient mice had impaired glucose metabolism and a decreased exercise tolerance (17). The researchers examined the long term effects of exercise by fattening normal mice and autophagy-deficient mutant mice, which gave both groups a form of diabetes. After two months of daily treadmill exercise in both groups, the normal mice were able to reverse their diabetes through physical training. Training also lowered cholesterol and triglyceride levels. These benefits were not seen in the autophagy-deficient mice. Thus, it was concluded that autophagy is required for sustained exercise and there are relationships between glucose

utilization, autophagy, and diabetes. More human research is needed to confirm these results because the understanding of the relationship between exercise and autophagy is still not completely elucidated.

Autophagy, the cell's recycling center

Autophagy was first described in 1962 through the use of transmission electron microscopy (TEM) in glucagon treated rat livers (2). In this communication, the authors designated stages of lysosomal breakdown of mitochondria in which they described the presence of cytoplasmic microbody particles 0.4 to 0.6 microns in diameter distinct from the lysosome. Ashford et al. described these particles "as though each lysosome, when first formed, is a packet of cytoplasm segregated from the surrounding viable cytoplasm by a membrane or membranes" (2). The authors then stated that "the cell leads to the development of foci of physiologic autolysis and such areas are automatically surrounded by a membrane, possibly to shield the rest of the cell from the general spread of the degradative process" (2). Although the authors did not term this process "autophagy", the description of a membrane formation around cytoplasmic compartments is consistent with the formation of the autophagosome.

Since that time about 35 autophagy-related genes (ATGs) and proteins (Atgs) have been identified. These ATGs orchestrate multiprotein complexes that act in concert to degrade and recycle damaged, unwanted, or redundant cell membranes and organelles including mitochondria, endoplasmic reticulum, and peroxisomes (8, 15, 40). Autophagy ensures cytoplasmic homeostasis enabling cells to digest their own cytosol and has many important roles including; innate and acquired immunity, inflammatory pathology, and T and B cell homeostasis (10). Autophagy is also thought to be an immunological effector

and regulator of immunity through sampling, digestion, and presentation of peptides against intracellular microbial pathogens (10, 28, 40).

Autophagy is a dynamic process that plays an important role in the turnover of intracellular proteins and organelles in the event of nutrient deficiency (8, 15). Under normal conditions, the intracellular pool of amino acids is preserved by the proteasome that constantly degrades cytoplasmic proteins (22). In contrast, autophagy maintains the amino acid pool in times of starvation and stress (22). Sometimes termed autophagocytosis, autophagy is recognized as a cell survival mechanism during periods of nutrient deprivation where non-critical organelles and bulk degradation of cytoplasmic proteins are simplified to building blocks for the formation of alternative cell energy (35). The autophagy process occurs in three general phases: (1) the target molecule is engulfed by a double-membrane vesicle called an autophagosome, (2) the autophagosome carrying the target molecule is shuttled and presented to a lysosome, and following presentation of the target molecule, (3) the autophagosome and contents are degraded by lysosomal proteases (15).

Autophagy Marker LC3-II

Microtubule associated protein 1b light chain 3 (LC3/Atg8) is a widely used marker of autophagy (30). LC3-II is most commonly used as a marker of autophagy because its lipidation and specific recruitment to the autophagosome allows staining of this protein and increases its electrophoretic mobility on gels as compared with LC3-I (3, 22). LC3 is an essential mammalian ortholog protein that functions to mediate membrane fusion of the autophagosome by fusing the autophagosome membrane for sequestration of large mitochondria and protein aggregates (43, 44). LC3 can be detected by

immunoblot analysis in the form of LC3-I (14 kD) and LC3-II (17 kD) (30). LC3-II has been shown to highly correlate with the number of autophagosomes and is a good indicator of autophagosome formation (30). LC3-II is often problematic to interpret because it is degraded during autophagy. To avoid this problem, LC3-II can be measured in the presence and absence of lysosomal protease inhibitors (3, 30).

Stimulation of autophagy

Production of LC3-II can be stimulated by starvation conditions (30). In mouse embryonic fibroblast (MEF) cells it was found that during short starvation periods the amount of LC3-I decreased while the amount of LC3-II increased (29). A chief regulator of autophagy is the kinase mTOR (35). The protein mTOR is important for regulating the nutrient hormonal signaling network and is involved in different pathological responses including obesity, diabetes, and cancer (9). When activated, mTOR suppresses autophagy induction. Known pathways of mTOR activation occur through phosphatidyl-inositol 3 kinase (PI3K), the insulin protein kinase B (Akt) pathway, feeding (amino acid availability), and mitogen activated protein kinase (MAPK) signaling (35). A well-known activator of mTOR is the growth factor Akt (also known as PKB) that is activated in the presence of circulating insulin (46). Following exercise, insulin is known to increase to maintain glucose homeostasis (39). Mechanistically, by activation of the insulin/Akt pathway, it may be possible that insulin activates mTOR via Akt, which would in theory, then inhibit the activation and actions of autophagy.

It is accepted by most researchers that pathways that inhibit mTOR promote autophagy, and pathways that stimulate mTOR prevent autophagy induction. Amino acids have long been known to regulate autophagy; however, only certain amino acids are

capable of controlling autophagy and their actions are highly specific (35). It is also thought that autophagy may be activated through mTOR-independent pathways (21, 31). Conversely, the induction of autophagy occurs when mTOR is inhibited through the AMP-protein kinase (AMPK), FKBP12 (rapamycin), and p53 signaling pathways (35).

Caloric restriction (CR) is a known stimulator of autophagy and has been documented to have positive effects on the median and maximum life span of rodents (4, 8). More than 60 years of research has shown that dietary restriction is the only nutritional intervention that consistently extends the lifespan and health of animals and may counteract age-related changes in tissue physiology (4). Possible mechanisms of increased longevity in humans include: CR, exercise, and genetic disruptions of the insulin and GH/IGF-1 axis to lower insulin and IGF-1 levels and increase macroautophagy (4). CR has been shown to delay the onset of age-associated diseases, reduce metabolic rate and oxidative stress, and decrease blood sugar and increase insulin sensitivity (4). After feeding, an increase in plasma glucose, amino acids, insulin, and IGF-1 levels occur. Feeding then results in endocrine and metabolic changes and may be responsible for suppression of the autophagy process and the slow turnover rate of long lived molecules (4). Without autophagy, an accumulation of altered membranes, mitochondria, and peroxisomes could enhance free radical production, cell injury, and start a cycle of irreversible cell changes and decreased life span (4).

Cell culture models are extremely useful in investigating the mechanisms of cell biology. A human lung epithelial carcinoma cell line (A549) infected with an adenovirus directing expression of heat shock protein-70 (HSP70) was used to determine the effect of starvation conditions on autophagy. Two hours post starvation, control cells (infected

with a control adenovirus) had a significant increase in LC3-II indicating an increase in autophagy (13). Cells containing HSP70 overexpression prevented starvation-induced autophagy, suggesting that HSP70 may regulate autophagy (13). Similar effects of HSP70 overexpression on autophagy were also seen in human non-small cell lung cancer (H1299) and colorectal adenocarcinoma (Caco-2) cells (13). These experiments consistently show that there is a connection and regulatory control by heat shock protein on autophagy.

Exercise and autophagy

Physical exercise has been documented to have a positive effect on lifespan (24). Catabolic pathways are accelerated during exercise to supply the energy and substrates to muscle for continuation of contractions (22). As stated previously, autophagy is an intracellular catabolic process that results in the autophagosomic-lysosomal degradation of cytoplasmic matter (34). Autophagic recycling helps cells meet energy demands and could account for exercise's benefits for humans that participate in regular physical activity (23). Exercised voluntarily, running rats live longer than freely eating sedentary controls and are lower in weight. This is thought to be due to enhanced autophagy and better cell repair (4). Early evidence also indicated that strenuous exercise induced autophagy. Recent studies suggest that there may be an exercise-autophagy parallel. One theory of this parallel suggests that exercise mediates its cardioprotective effects in part by induction and up regulation of autophagy (15).

Although the role of exercise on stimulating autophagy has been described in mice, research on humans is extremely limited and even less is known about the effect that moderate physical exercise has on autophagy (4). In older obese women (65.8 ± 6.2

years; 36.1 ± 4.7 kg/m^2), a study was conducted to explore the effect of weight loss plus an exercise program on the quality control mechanisms autophagy, apoptosis, and other cellular pathways in skeletal muscle (44). The study reported a threefold increase in mRNA levels of autophagy regulators LC3B, Atg7, and lysosome-associated membrane protein-2 (LAMP-2) in weight loss plus exercise program subjects after a 6 month training program (44). It is thought that weight loss plus exercise stimulates cellular quality control mechanisms which can contribute to improved overall muscle health and function during aging (44). Recent data from our lab shows that following intense exercise (60-minute treadmill run at 70-80% of VO_2max in a warm, 30˚C, environmental chamber) autophagy is significantly increased in human PBMCs (13). In the same study, it was also shown that glutamine supplementation for 7 days prior to exercise associated with a core temperature of $\geq$ 39˚C resulted in an increase in HSP70 and prevented the exercise-induced increase in autophagy seen without supplementation (13). This study was the first to show that exercise associated with hyperthermia-induced autophagy in humans and the autophagy response could be prevented by glutamine-induced HSP70. These data indicate that autophagy is involved in maintaining energy and protein homeostasis and may be under the regulatory control of the heat-shock protein response (13).

Inflammatory response and autophagy

It is generally accepted that immunity can be improved by performing regular bouts of moderate intensity exercise, while frequent bouts of vigorous exercise may depress the immune system in athletes and may increase the risk of infection (26). Inflammation is a complex process that involves a series of events. One pathway in

which exercise may alter immunity is through the action of cytokines (36). Cytokines are proteins involved in the inflammatory process and are produced very early in the response to stresses that target tissues such as endothelial cells lining vascular walls (14). Acute aerobic exercise is known to increase cytokine production when compared to the resting state (26). One hour following 60 minutes of cycling at 95% of maximal steady state, an increased number and percentage of CD8 cells expressing IL-2, TNFα, IL-6, IL-4, and IL-10 was observed (26). This study showed that acute high intensity cycling exercise led to increased cytokine expression.

Proinflammatory cytokines induce inflammation and include cytokines characterized as being inducible and may belong to families such as IL-1, IL-6, and TNFα (14). Excessive inflammation can be detrimental and may lead to pathology (14). Proinflammatory cytokines include TNFα, IL-1, IL-6, chemokines, and are produced by macrophages (14). However, abundant evidence also suggests that proinflammatory cytokines are very important for the activation of acquired immune response that is usually beneficial (14). IL-6 is a major product of IL-1 or TNF-stimulated cells and is an important β-cell differentiation factor (14). IL-6 also has anti-inflammatory characteristics, such as the capacity to downregulate TNFα production (14).

Autophagy is emerging as a process of high importance for innate and adaptive immunity responses (5, 7). A recent study assessed the effect of autophagy on the production of proinflammatory cytokines in human cells (7). The inhibition of autophagy in PBMC cells was found to increase IL-1β (also known as IL-1) production after stimulation, while TNFα was significantly reduced by agents that inhibited autophagy (5, 7). The effect of autophagy on cytokine production was at the transcriptional level and is

thought to involve the inhibition of p38 mitogen activated protein kinase (MAPK) phosphorylation. The authors concluded that autophagy modulates the secretion of proinflammatory cytokines in human PBMCs through an inflammasome-independent pathway (7). Cytokine IL-6 which is known to be released from working muscle was not measured in this study.

Exercise is important for longevity through cell homeostasis

Regular physical activity at any level compared to inactivity is beneficial in that it reduces the probability of developing diabetes, high blood pressure and arthritis in individuals in fair or poor health (20). In New Mexico, the 2008 prevalence rates of diagnosed diabetes for adults estimated that both Hispanic and American Indian/Alaska Native adults are 1.6 times more likely than White Non-Hispanic adults to have diagnosed diabetes. These statistics and those cited above demonstrate the high need for exercise-based health studies such as ours in the state of New Mexico. This study of autophagy and physical activity has important health implications in the study of disease and can lead to the understanding of how sub-maximal endurance exercise may increase autophagy and, therefore, maintain cell homeostasis and integrity.

Purpose and hypotheses

The literature provides a strong rationale and reveals the need for further investigation of the relationship between immune function, autophagy, and exercise. Exercise has recently been shown to be a stimulus of autophagy (17). Autophagy can be induced by physical activity through exercises ability to cause energy deprivation, by its effect to stimulate protein unfolding, or by increasing oxidative stress (12, 29, 33). Currently, there are no studies that have examined the intensity of exercise required to

increase autophagy in humans. Recent preliminary studies from our lab show that there are significant increases in autophagy markers in human PBMCs 2 and 4 hours post exercise following high intensity treadmill running (70-80% VO_2max) for 60 minutes in a warm (30˚C) environmental chamber (13). That study also showed that increases in HSP70 expression decreased exercise-induced autophagy (13). We intend to investigate if 60 minutes of endurance exercise at a moderate intensity (50% VO_2max) will also induce autophagy and HSP70 when compared with high intensity treadmill running (70-80%VO_2max) of a similar duration in a warm environment.

Measuring autophagy and HSP protein expression levels over different post-exercise time points will provide insight about possible interactions between these two cellular systems. We examined the early time course of autophagy markers, heat shock protein, cytokine 1L-6, insulin, and related autophagy proteins in PBMCs following endurance exercise. To describe this time course we collected blood samples pre-exercise, post-exercise, 30 minutes post-, 1 hour post-, 2 hours post-, and 4 hours post-exercise. Autophagy has important modulatory effects on inflammation, although exercise-induced autophagy has yet to be confirmed as a modulator of inflammation in human PBMCs. We also examined the modulatory effects of inflammation responses following endurance exercise and tested for relationships with the changes in autophagy by measuring IL-6.

Purposes of this Study

The purpose of this study is multi-fold; specifically, this study was designed to:

1. Document the early time course of exercise-mediated autophagy in human PBMCs following two bouts of endurance exercise at different intensities.

2. Investigate the relationship between heat shock protein expression and autophagy markers in PBMCs at several time points following exercise.
3. Assess the effect of exercise-induced IL-6 on induction of autophagy.
4. Evaluate involvement of insulin and Akt in autophagy regulation.

Hypothesis

In this study we will test the following hypotheses:

1. 60 minutes of endurance running at 70% of VO_2max in a warm environment will initiate early catabolic autophagy-mediated signaling mechanisms when compared to baseline levels.
2. There will be no significant differences in autophagy signaling between baseline values and those following 60 minutes of endurance running at 50% of VO_2max in a warm environment.
3. There will be no significant differences in autophagy signaling between following 60 minutes of endurance exercise at 50% of VO_2max in a warm environment when compared to the same time points following high intensity endurance running.
4. There is a negative relationship between LC3-II and HSP70 expression following high intensity endurance running.
5. There is a negative relationship between LC3-II and HSP70 expression following moderate intensity endurance running.
6. IL-6 cytokine production by skeletal muscle will not significantly decrease autophagy at high intensity exercise when compared to rest.

7. IL-6 cytokine production by skeletal muscle will not significantly decrease the autophagy at moderate intensity exercise when compared to rest.
8. Decreased plasma insulin levels will activate autophagy through inhibiting the Akt signaling pathway following high intensity exercise.
9. Decreased plasma insulin levels will activate autophagy through inhibiting the Akt signaling pathway following moderate intensity exercise.

Scope of Study

Based on *apriori* power analysis indicated by our pilot studies, we needed 8 subjects (15 recruited for anticipated attrition) to complete two endurance treadmill-based exercise trials. All subjects were assessed for body fat estimation, height, weight, VO_2max, and completed a health history questionnaire, HIPPA authorization form, and informed consent. Subjects were instructed to avoid moderate to intense physical activity 12 hours before testing, alcohol, caffeine, fasting, dieting, supplements or drugs that alter metabolism, and changes in diet before participation. Male and female endurance trained subjects ages 18-40 years old from the Albuquerque, NM area participated in this study. Each subject completed a 50% VO_2max exercise trial for one hour and a control exercise trial for 1 hour at an intensity of 70% VO_2max, separated by a minimum of a two week washout period. Both exercise trials took place in a warm-environmental chamber set to 30˚C. The order of exercise intensity was randomized. Western blot protein analysis and ELISA was used to measure LC3-II protein, Akt protein, HSP70 protein, insulin, and IL-6 protein. Insulin and IL-6 cytokine were adjusted for changes in plasma volume.(11, 41) Quantitative real-time PCR was used to measure LC3B and HSP70 mRNA.

Assumptions

It was assumed that LC3-II was a reliable measurement of autophagosome formation and thus, a good marker of autophagy. It was also assumed that differences found in laboratory results were due to variation caused by the study intervention, and not due to poor lab or data collection techniques.

Limitations

The authors of this study recognize there are limitations to the design. In this study we measured the effects of exercise intensity on autophagy, heat shock response, insulin levels, and cytokines. We measured indirect markers of autophagy autophagosome formation LC3-II, and inhibitory proteins Akt and Insulin. Another limitation is that we measured autophagy in PBMCs and not skeletal muscle tissue following exercise. These findings may not truly reflect changes seen due to the continuous contractile action of exercise in working tissue but changes due to circulating cytokines or protein signaling molecules.

Statistically, one limitation is that we did not randomly assign subjects to groups because we wanted to recruit subjects that were capable of competing exercise at a high intensity for a long duration (1h). We also did not control the subject's diet, past pharmaceutical and drug use, lifespan exercise regimen, or biological variability; however, subjects were advised to avoid intense exercise, caffeine, alcohol, and metabolic enhancers the day before the exercise trials.

Because of the strenuous exercise protocol needed to induce autophagy, we tested endurance trained athletes. It is possible that this population may already maintain an elevated level of heat shock protein. All subjects completed exercise in the same season

(summer: May and June) to avoid variation in heat-shock proteins associated with seasonal changes in temperature. This study was designed to be a within-subject design where each subject served as their own control. A two week wash-out period between exercise trials was employed to avoid carry over effects of autophagy stimulation. Exercise intensity was randomized to avoid a possible training adaptation or order effect.

Significance of Study

Among the top ten leading causes of death in New Mexicans (2010), according to the NM Department of Health Statistics Annual Report, were five diseases associated with physical inactivity. Cancer, heart disease, stroke, Type II diabetes mellitus, and Alzheimer's disease are all documented to be modifiable by exercise and/or can be improved by regular physical activity. When compared to national statistics, New Mexico exceeds the national rate of diabetes-related deaths by more than 25%. In 2012, 50% of American Indian and 40% of Hispanic third-graders in New Mexico were either overweight or obese. These health outcomes reveal a high need for research in the areas of physical activity and the physiological systems that are responsible for the health benefits of exercise. Studies on immune function, exercise, inflammation, and autophagy are limited in human subjects. An understanding of the ability of exercise to acutely alter autophagy may lead to improved health outcomes and longevity.

Specifically, we believe this study will provide insight into the exercise intensity required to induce autophagy and the role of exercise in stimulating autophagy. It is known that exercise elicits an acute inflammatory response and autophagy modulates secretion of proinflammatory cytokines. This study will also provide information about the circulating "exercise cytokine" IL-6 and the relationship with autophagy. It is also

known that autophagy is stimulated by starvation conditions. By studying the insulin/Akt pathway following endurance exercise we may reveal the mechanism in which autophagy may be stimulated by exercise.

Definitions

ACSM and CDC Physical Activity Recommendations (2008) – American adults aged 18-65 years should continue to accumulate at least 30 minutes of moderate-intensity aerobic activity 5 days per week OR engage in 20 minutes of vigorous activity 3 days per week. Strength training at least twice weekly consisting of 8-10 exercises for at least 1 set of 8-12 repetitions each.

Autophagy – or autophagocytosis, is derived from the Greek word for 'self-eating'. It is the basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the lysosomal machinery.

Cytokines – signaling molecules that refer to immunomodulating agents such as interleukins and interferons involved in the inflammatory process

Heat-shock proteins (HSPs) – proteins induced by heat shock or other stressors. Named according to their molecular weight, heat shock proteins function in the refolding of damaged proteins

The mammalian target of rapamycin (mTOR)- also known as mechanistic target of rapamycin or FK506 binding protein 12-rapamycin associated protein 1 (FRAP) is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription.

Moderate-intensity physical activities – brisk walking, cycling, or any equivalent ranging from 3.0-5.9 METs

Percentage of VO_2max – used to determine exercise intensity by calculating the percent of oxygen consumption determined from a maximal graded exercise test.

Peripheral blood mononuclear cells (PBMC) – Any blood cell that has a round nucleus (i.e. a lymphocyte, a monocyte, or a macrophage). These blood cells are a critical component in the immune system to fight infection and adapt to intruders.

Rapamycin – also called Sirolimus, is an immunosuppressant drug used to prevent rejection in organ transplant. The mechanism of action is by inhibiting the mammalian target of rapamycin pathway by directly binding the mTOR Complex 1 (mTORC1).

Vigorous-intensity physical activities – include running, cycling, or any equivalent greater than 6.0 METs

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CHAPTER II

This chapter presents a review article entitled “The Breakdown of Autophagy in Exercise” which is formatted for the *British Journal of Sports Medicine*. It is authored by Kathryn Rosie Lanphere, Micah Zuhl, Christine Mermier, Suzanne Schneider, Ann Gibson, Karol Dokladny, and Pope Moseley.

The Breakdown of Autophagy and Exercise

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ABSTRACT

The benefits of regular physical activity can be, in part, explained by the catabolic degradation pathway of autophagy. Exercise-induced autophagy is a newly researched avenue that has provided much insight about the importance of autophagy in regular physical exercise, disease, glucose homeostasis, and general health. Endurance and resistance exercise provide many stress stimuli that change cellular composition and lead to physiological adaptations. Autophagy has a role in both resistance and endurance exercise, although more studies on endurance exercise are available. Studies on endurance exercise-initiated autophagy generally show increased autophagy following exercise. This review discusses our current understanding of the principle functions of autophagy and describes how alterations by acute exercise and chronic training interventions influence autophagy.

INTRODUCTION

The study of exercise-induced autophagy is a fairly novel and quickly growing field. Exercise provides a stimulus that induces skeletal muscle adaptation and requires the addition as well as clearance of cellular components.[1, 2] Much is known about the biosynthesis of contractile proteins and mitochondrial biogenesis adaptations to exercise training, whereas much less is known about the clearance and breakdown of long lived and damaged macromolecules resulting from these adaptations. Macroautophagy is an essential catabolic process that contributes to the degradation, clearing, and recycling of protein aggregates, aged cell components, intracellular pathogens, and excess or damaged organelles.[3] Macroautophagy, which will now be referred to as autophagy, contributes to cellular homeostasis and also promotes widespread health benefits including protection from sarcopenia, type-II diabetes, some cancers, inflammatory disorders, neurodegenerative disease, infection, and aging.[2, 4-12] Several studies have confirmed the effects of exercise on autophagy induction.[1, 2, 4, 5, 7, 9, 10, 13-16] These studies were typically performed in animal models. Human research in autophagy is becoming more prevalent.

Exercise is known to elicit cellular disturbances that disrupt metabolic homeostasis.[17, 18] This disruption leads to the induction of several cellular maintenance processes and protein synthesis.[19, 20] Pathways of autophagy induction occur following stress and may be induced by the same pathways associated with cellular disturbances that occur during exercise. Factors such as protein turnover, mechanical stress, availability of nutrients, and induction of growth factors are all associated with muscle atrophy and hypertrophy signaling following exercise.[21-23] Similar factors

have also been investigated in the autophagy-auto-induction pathway and may have relationships with autophagy regulation.[19] The inclusive effects of exercise on cellular mechanisms remain incomplete and are currently under extensive investigation. The purpose of this review is to describe the process and regulation of macroautophagy (autophagy), summarize recent findings that are relevant in exercise research, and name the possible avenues that autophagy and exercise may share in clinical settings.

The "Breakdown" of Autophagy

Autophagy, which literally means "self-eating", is a catabolic pathway that traffics cellular components to the lysosome. In cells, the lysosome is responsible for breaking down a wide range of cellular material such as damaged proteins, organelles, and cellular constituents marked for degradation. Autophagy describes the process whereby bulk cytoplasmic components are sequestered in a double-membrane structure known as the autophagosome and delivered to the lysosome as cargo. Other forms of autophagy differ by the means they use to deliver cargo to the lysosome and, by name, are called microautophagy and chaperone-mediated autophagy.[24] The morphological hallmark of macroautophagy is the formation of a double-membrane cytosolic vesicle, the autophagosome, which sequesters cytoplasm and delivers it to the lysosome.[25] Many steps upstream from autophagy induction must be initiated prior to the conditional assembly of the autophagosome. The formation of the autophagosome can be broken down into 4 general phases: (1) a double membrane phagopore is created by induction of autophagy related genes (Atg), (2) the phagopore is elongated into an autophagosome in which the target molecules are sequestered, (3) the autophagosome carrying the target molecules is shuttled to and combines with a lysosome to create an autophagolysosome,

and (4) the autophagolysosome and contents are degraded by lysosomal proteases and released as nucleotides for protein synthesis.[10, 26]

Autophagy Machinery Assembled for Breakdown

Upon induction of autophagy, autophagy related genes (Atgs) that assemble components of the autophagosome are activated.[9] Figure 1 depicts autophagosome formation and the involvement of several autophagy-specific genes following auto-induction. The preliminary steps of autophagosome formation involve the association of Atg13 with Atg1 kinase. This association forms a complex with Atg17 to create a phagopore.[9] The association of dephosphorylated Atg13 and Atg1 then stimulate a catalytic reaction to the conditional formation of the autophagosome.[9] The conjugation of Atg12-Atg5 and the Atg8-phosphatidyl ethanolamine (PE) systems are also necessary for autophagosome formation.[27] Upon induction of these systems, the process of autophagosome vesicle elongation begins. Elongation involves the covalent conjugation of Atg12-Atg5, with the aid of Atg7 and Atg10.[9] Next, conjugation of Atg8-PE to microtubule-associated protein-1b-light-chain-3 (LC3) occurs to complete the vesicle elongation phase.[9] Chronologically, LC3 is converted from the soluble form LC3-I, to the autophagic vesicle associated form LC3-II with the aid of intermediate proteins PE, Atg3, Atg4, and Atg7.[9] The conversion of LC3-I to LC3-II is ultimately required for membrane expansion and is a good indicator of autophagosome formation.[9, 28]

Following membrane expansion the autophagosome undergoes maturation by fusion with a lysosome and lysosomal associated membrane protein (LAMP2).[9] The marriage of the autophagosome and lysosome is termed the autophagolysosome and provides the hydrolases necessary to catalyze the hydrolysis of the autophagosomal

contents.[9] The contents are then released and return to the cytoplasm of the cell to be recycled as building blocks for other cell processes and functions. It is thought that autophagy recycling helps cells meet energy demands during exercise and may also increase longevity by reducing the number of aged cytosolic components when stimulated by physical activity.[14] The initial steps of autophagy machinery assembly are conditional under the circumstance when the major regulatory protein mTOR is inhibited. Autophagy can also be induced independent of mTOR through other autophagy-related genes such as Beclin-1, also called Atg 6.[29] Beclin-1 is essential for vesicle nucleation and functions as part of a multi-protein complex involving class III phosphatidyl-inositol 3 kinase (PI3K) and vacuolar protein sorting 34 (Vps34).[29]

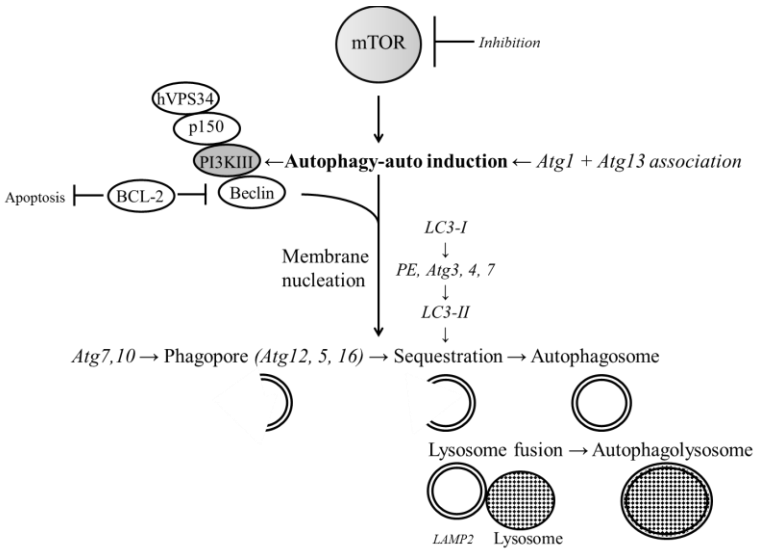

Figure 1. Autophagosome formation. Autophagy-related genes (*Atg*) activate components of the molecular machinery responsible for autophagy action. These genes control a number of aspects of the autophagic process including induction through Beclin-1 (Atg6) and autophagosomal vesicle formation through Atg12-Atg5 and LC3-I (Atg8). Beclin-1-dependent autophagy can be inhibited by binding of Bcl-2 to Beclin. Autophagy and apoptosis are connected and a crosstalk exists between the two through Bcl-2. Grey ovals represent kinase proteins. Black lines show inhibition of pathways and black arrows show activation of the pathway. This figure was modified from Cell Signaling Technologies.

Autophagy Regulation by mTOR

Autophagy is regulated by multiple signal-transduction pathways that are also commonly studied in exercise and skeletal muscle hypertrophy.[19] Activation of autophagy generally occurs during times of nutrient deprivation but can also be stimulated during the differentiation of cells, fetal development, neurodegenerative diseases, infection and cancers.[30-33] The mTOR signaling pathway is known as the major pathway regulating autophagy and suppresses autophagy when active. mTOR is a complex protein that integrates signals from the environmental stimuli and is a sensor for energy balance in the cell.[20] This energy sensing molecule is important for governing protein turnover in exercising muscle and favors cell growth by inhibiting proteolytic system components.[20, 34] The main signaling pathways of autophagy inhibition through mTOR occurs through phosphatidyl-inositol 3 kinase (PI3K), protein kinase B (Akt), and mitogen activated protein kinase (MAPK) signaling.[35] It is widely accepted that the preponderance of autophagy activation is conditional upon the inhibition of mTOR. Conditions such as the lack of nutrient unavailability and hypoxia inhibit mTOR activation. Inhibition of mTOR may occur through signaling by the AMP-protein kinase (AMPK) and p53 pathways.[35] A diagram of autophagy signaling via the mTOR pathway is presented in Figure 2.

Endogenously, mTOR can be presented in two complexes called mTORC1 and mTORC2. The mTORC1 protein complex is made up of the proteins mTOR, raptor, GβL, and inhibitory protein PRAS40.[36] Active mTORC1 interacts with ULK1 (Atg1) and inactivates autophagy by phosphorylating ULK1. Under starvation conditions or treatment with rapamycin, the inactivation of mTOR results in the dissociation of the

mTORC1 complex which dephosphorylates ULK1 and activates autophagy.[21] Exploring the similarities between mTOR inactivation and autophagy during exercise may reveal an autophagy-exercise parallel and complement the growing knowledge regarding the benefits of human exercise.

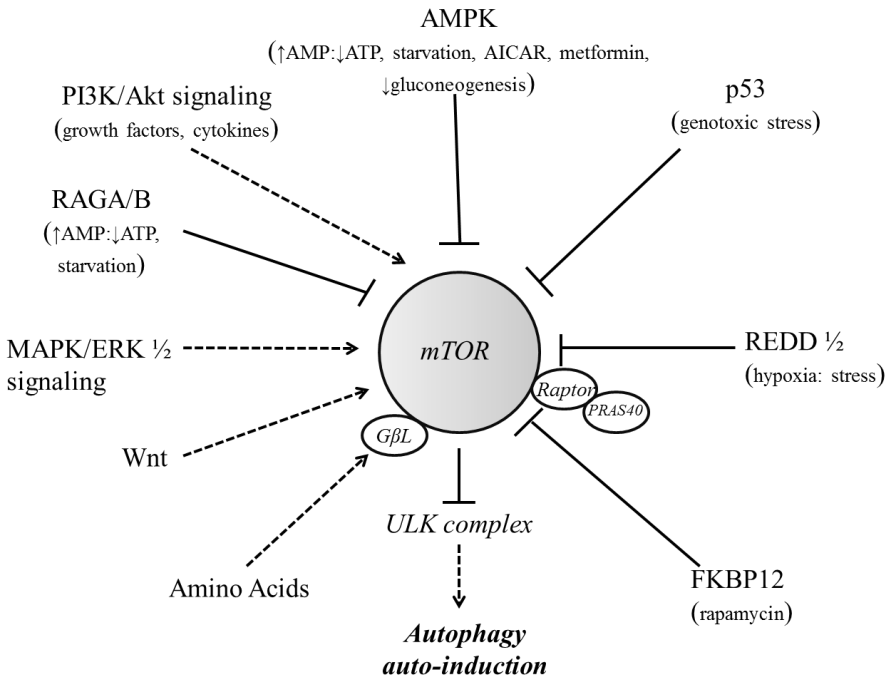

Figure 2. The mTOR signaling summary**.** The kinase mTOR is a critical regulator of autophagy induction. Activated mTOR (pathways: Wnt, PI3K/Akt, MAPK/ERK1/2, Amino Acids) suppresses autophagy, and negative regulation of mTOR (pathways: AMPK, REDD1/2, RAGA/B, FKBP12, p53) promotes autophagy. Upon inhibition of mTOR, ULK complex activates autophagy auto-induction. Lines show inhibition of mTOR and dotted arrows show activation of mTOR. This figure was modified from Cell Signaling Technologies.

Autophagy is Increased Following Endurance Exercise

Endurance exercise is a newly defined stimulus that induces autophagy *in vivo*.[2, 16, 37] Catabolic pathways are accelerated during exercise to supply the energy and substrates to muscle for continuation of contractions.[9] Exercise thus creates conditions

that favor autophagy breakdown. A recent letter in *Nature* describes studies in mice that show the induction of autophagy following acute endurance exercise in skeletal and cardiac muscle of fed mice.[2] He et al. [2] created a transgenic mouse model that expressed a green fluorescent protein (GFP)-labeled marker of autophagosomes, GFP-LC3 (also known as MAP1LC3). Mice completed treadmill exercise for 80 min (~900m) and puncta was observed for GFP-LC3 in several muscles and organs involving energy homeostasis. Autophagosome (GFP-LC3) numbers were increased after 30 min (~300m) of running and reached a plateau at 80 min in both cardiac and several skeletal muscle groups including: the vastus lateralis, soleus, tiabialis anterior, and extensor digitorum longus. Other mouse studies have also reported increases in autophagy following endurance exercise. In cardiac muscle of rats, a single bout of running exercise for 30 min resulted in a "biphasic" change in autophagy with an initial decrease observed immediately after exercise and a subsequent increase 1 h thereafter.[7] In other rat models, treadmill exercise has been shown to induce autophagy in peripheral tissues (muscle, liver, pancreas, adipose tissue) and the brain.[14]

Conversely, some endurance exercise studies have shown decreases in autophagy. Kim et al. reported a decrease in autophagy marker LC3-II 3h, 6h, and 12h post-exercise in murine skeletal muscle following 50 min of running.[9] It is known that caloric restriction, and not feeding, increases autophagy. These mice were provided with chow and water *ad libitum* and were not indicated to be fasted.[9] Swim training studies on diabetic rats have also reported decreased baseline levels of autophagy and increased muscle mass when compared to diabetic non-exercised controls.[13] Lee et al. [13] contributed the loss of muscle mass in diabetic rats to the hypercatabolic metabolism

associated with autophagy in diabetes. Consequently, the feeding state, altered health and physiological state of the animals, non-human models used for analysis, and time point at which autophagy was measured should be taken into consideration.

Although the role of exercise on stimulating autophagy has been well described in mice, research on human models of autophagy is limited and even less is known about the effects of moderate-intensity physical exercise on autophagy. In human studies, ultraendurance exercise was shown to upregulate autophagy genes in skeletal muscle.[37] This study showed that following a 200 km race, LC3B mRNA was increased by 103% 3h following an ultramarathon.[37] This extreme case of autophagy upregulation is not only seen following these types of punishing exercise but can also be observed following lower intensity exercise bouts. Recent data from our lab shows that following high-intensity exercise (60-minute treadmill run at 70-80% of VO_2max in a warn environmental chamber, 30˚C) autophagy is significantly increased in human PBMCs.[38] In the same study, it was also shown that glutamine supplementation for 7 days prior to exercise associated with a rectal temperature of ≥ 39˚C resulted in an increase in HSP70 and prevented the exercise-induced increase in autophagy seen without supplementation.[38] Dokladny et al. published the first study to show that exercise associated with hyperthermia induced autophagy in humans and that the autophagy response could be prevented by glutamine-induced HSP70. These data indicate that autophagy is involved in maintaining energy and protein homeostasis and may be under the regulatory control of the heat-shock protein response.[38]

Autophagy is Decreased Following Resistance Exercise

Resistance exercise (RE) is a common intervention used to attenuate the loss of lean muscle mass and strength in young and older adults.[15] Skeletal muscle is a major site of metabolic activity, is the most abundant tissue in our body, and provides a huge source of amino acids to be utilized for energy production.[39] Mouse studies have shown that blockage of autophagy in skeletal muscle results in atrophy.[40] Masiero et al. demonstrated that autophagy flux is important in preserving muscle fiber integrity and muscle mass in general.[41] Protein catabolism following RE has been shown to be regulated through the autophagy-lysosomal system.[42, 43] The Akt-FoxO3A signaling pathway is commonly associated with protein degradation following RE and has recently been shown to regulate autophagy genes LC3 and GABARP.[44-46] Following an acute bout of RE, 8 sets of 10 repetitions at 70% one repetition maximum, autophagy marker LC3B-II has been shown to significantly decrease 6 and 24 hours post-exercise in young individuals when compared to baseline.[15] In older individuals, the same decrease in autophagy was seen with an additional decrease 3 hours post-exercise.[15] Also, the total content of Beclin-1, an upstream regulator of autophagy, did not change in this study and was significantly lower in younger participants compared with older participants during all time points. This is one of few recent studies that illustrate a potential downregulation of autophagy following RE in humans and rats.[42, 43] Following resistance exercise, mTOR has been shown to stimulate transcription of downstream proteins associated with muscle hypertrophy.[47] One study showed that autophagy is important to maintain skeletal muscle integrity.[45] Autophagy may be stimulated during resistance exercise to cope with the stress of diminished energy substrate, oxidative stress, and hypoxia but is then down-regulated following exercise by other signaling and growth factors such as

mTOR, to facilitate muscle rebuilding. Autophagy studies in resistance exercise are the most limited and should be examined with careful consideration and timing of muscle tissue collection. The complete understanding of how RE affects autophagy is still under investigation.

Autophagy is Required for the Beneficial Long-term Effects of Exercise Training

Regular physical activity and exercise training have been shown to have numerous benefits including improved health outcomes in diseased and healthy individuals.[48-50] Increased autophagy function has been shown in exercise training studies. In older obese women (65.8 ± 6.2 years; 36.1 ± 4.7 kg/m^2), a training study was conducted to explore the effect of a weight loss plus exercise program on the quality control mechanisms of autophagy, apoptosis, and other cellular pathways in skeletal muscle.[5] The study reported a threefold increase in mRNA levels of autophagy regulators LC3B, Atg7, and lysosome-associated membrane protein-2 (LAMP-2) in weight loss plus exercise program subjects after a 6-month intervention training program.[5] It is thought that weight loss plus regular exercise training stimulates cellular quality control mechanisms that contribute to improved overall muscle health and function during aging.[5]

Lira et al. investigated whether exercise training alters basal levels of autophagy and whether autophagy is required for skeletal muscle adaption to training.[1] Mice were exercised daily on a treadmill for 4 weeks. Long-term voluntary running resulted in increases in basal autophagy and expression of autophagy proteins in tonic, oxidative muscle compared to muscles of either mixed fiber types or of predominant glycolytic fibers.[1] These findings suggest that endurance exercise training increases basal

autophagy and takes place if an enhanced oxidative phenotype is achieved.[1] This is also the first study to show that autophagy protein expression is dictated by contractile activity independently of enhancements in an oxidative phenotype. These researchers also trained mice heterozygous for autophagy protein Atg6. They showed that the heterozygous mice had an impaired endurance capacity and no increases in basal autophagy, mitochondrial content, or angiogenesis in skeletal muscle following training.[1] These studies show the importance of autophagy in exercise-training induced skeletal muscle adaptations and that impaired autophagy results in the encumbrance of prolonged exercise performance.

Autophagy and Glucose Regulation Important Factors for Continuing Exercise

Metabolic disorders are major burdens on health systems globally as almost two-thirds of adult Americans are overweight.[51] Many of these disorders are due to a sedentary lifestyle that results in excessive body fat. This excess is believed to fosters the development of comorbidities such as hypertension, dyslipidemia, cardiovascular disease, and diabetes.[52] Insulin resistance is a large contributor to metabolic disorders in which glucose homeostasis is disturbed. Physical activity has been shown to improve glucose and metabolic homeostasis.[17, 18] During exercise, mammals must undergo several metabolic changes to increase skeletal muscle glucose utilization. Increasing muscles' sensitivity to insulin and the redistribution of glucose transporters, such as GLUT4, is critical for exercise-stimulated glucose uptake and sustaining exercise.[53, 54] To investigate the role of exercise-mediated autophagy on exercise-stimulated glucose uptake, He and colleagues developed a mutant mouse that was deficient in stimulus (exercise- or starvation)-induced autophagy.[14] Mutant mice contained a knock-in mutation in BCL2 phosphorylation sites that prevented the disruption of the BCL2-

Beclin-1.[55] Mechanistically, exercise-induced autophagy *in vivo* is thought to involve the disruption of the BCL2-Beclin-1 complex.[2] BCL2 is an anti-apoptotic and anti-autophagy protein that inhibits autophagy through a direct interaction with autophagy protein Beclin-1.[55] The disruption of this complex is critical for stimulus-induced autophagy in mammals.[55] These mice, termed BCL2AAA, showed a decreased endurance capacity, impaired exercise-induced increase in insulin sensitivity, and an altered glucose metabolism profile during acute exercise.[2]

BCL2AAA mice also displayed impaired chronic exercise-mediated protection against high-fat-diet-induced glucose intolerance.[2] Lastly, the mice failed to increase plasma membrane GLUT4 localization in the vastus lateralis and soleus muscles following maximal or 80 min running exercise, respectively.[2] These mice also had a notable decrease in AMPK activation in these muscles.[2] AMPK is known to have a central role in localizing skeletal muscle glucose by enhancing GLUT4 transporter protein to the plasma membrane. Additionally, AMPK is thought to activate autophagy in response to low energy conditions by sensing the cytosolic ratio of AMP to ATP.[56] Interestingly, exercise also induced autophagy in other organs, such as the liver, pancreas, islet β-cells, adipose tissue, and was involved in glucose and energy homeostasis.[2] These data show the importance of autophagy in sustaining endurance exercise and muscle glucose metabolism. These studies also suggest that cellular autophagy function is partially required for AMPK activation during exercise.[2] Furthermore, the studies conducted by He et al. imply that there are important clinical implications of autophagy in the study of diseases such as diabetes and cardiovascular disease where glucose localization and utilization has been compromised.[2]

Recently it has been shown that physical exercise, a known modulator of glucose homeostasis, stimulates skeletal muscle autophagy. Protein kinase B (Akt) is proposed to regulate signaling responses stimulated by vigorous exercise such as the phosphorylation of mTOR thereby promoting cell progress, hypertrophy, and protein synthesis. In this pathway, protein kinase B/PKB (Akt) is stimulated by insulin which then acts to activate downstream targets by phosphorylation.[57] Akt signaling is known to suppress autophagy via activation of mTOR.[44] Recent findings indicate that endurance exercise results in distinct phosphorylation of Akt (p-Akt) to promote glucose transport and glycogen synthesis in skeletal muscle.[57] In humans, phosphorylation of many Akt related signaling proteins was found to occur around 30-60 min after cycling exercise for 1h at 70% of VO_2max in the fasted state.[57]

Autophagy is also important for Cardiac Muscle Homeostasis

Autophagy is particularly essential for the maintenance of protein homeostasis in cardiac muscle tissue's requiring protein turnover to maintain their adaptability.[40, 41] A recent study suggests that exercise mediates a cardioprotective effect, in part, by induction and upregulation of autophagy.[10] As previously mentioned, acute exercise has been shown to induce autophagy in cardiac muscle of fed mice.[2] In other studies, it was found that mice lacking A-type lamins (serves as a model for skeletal muscle dystrophy) had impaired autophagy due to aberrant mTORC1 pathway activity. mTORC1 inhibition by rapamycin restored autophagy and importantly, improved cardiac muscle function.[40] It is known that hyper-activation of mTORC1 is associated with cardiac hypertrophy in a variety of disease models.[40] mTORC1 regulates protein synthesis and decreases turnover of cell components through inhibition of autophagy.[40]

Interestingly, elevated mTORC1 signaling is associated with normally aging tissues in mice, including liver, hematopoietic stem cells and likely other tissues.[40] In a range of model organisms, impaired autophagy is associated with aging.[40] Rapamycin administered late in life slows aging and extends the lifespan in mice. Together, these findings point to the importance of links between mTORC1 signaling and autophagy in a range of diseases as well as in normal aging. With additional investigation, we may find that interventions targeting the mTORC1 pathway may have an even larger range of therapeutic uses than those already in place.[40] Generally, autophagy is noted to have cardioprotective effects and is greatly required for maintaining optimal myocyte function.

Implications of Autophagy in Clinical Settings

Autophagy plays a vital role in the quality-control function in the cell by promoting basal turnover of long-lived proteins and organelles and selectively degrading damaged cell components. This quality-control provides protection from a variety of diseases, including neurodegeneration, glycogen storage disorders, myopathy, cardiovascular disease, and type-II diabetes.[58, 59] A wide variety of stressors can induce autophagy including nutrient deprivation, growth factor withdrawal, oxidative stress, infection, and hypoxia.[60] Autophagy machinery components are mutated in many human diseases highlighting the significance of autophagy in human health.[24] Autophagy is also known to play a pro-survival role during disease and has received immense interest in discerning how to most effectively modulate autophagy to treat cancer.[24] There are also contexts in which overactive autophagy and autophagy induction are cytotoxic. It is for these reasons that the study of exercise-induced

autophagy in humans is extremely important and, in this light, regular exercise may induce the optimal autophagy dosage for disease prevention and control.

CONCLUSION

Autophagy can be stimulated by endurance and resistance exercise and plays an essential role in quality control, energy balance, and cellular homeostasis. The mechanisms of autophagy induction and regulation are important for discerning the complete welfare of a competent and operational autophagic system. Autophagic "breakdown" of cellular components is critical for continued longevity and could have future impacts on the study of human health. The detrimental effects of autophagic dysfunction can be extrapolated from existing literature on disease showing defective autophagy as pathogenic. Exercise studies are extremely important in establishing a more thorough understanding of the perturbations that occur in autophagic flux. Such an understanding will be crucial for the development of therapeutics that target autophagy.

Summary of New Findings

At the present time, the compilation of autophagy and exercise *in vivo* models are extremely limited. Important findings of *in vivo* exercise studies on autophagy are summarized in Table 1. Most exercise studies have targeted skeletal acquired via biopsy from human and animal samples. Baseline levels of autophagy can be stimulated by the stressors associated with continued muscle contraction; these stressors include factors such as hypoxia, ATP and energy deficits, reactive oxygen species, increased temperate, inflammatory cytokines, decreases in pH, and are currently under review.

Table 1. Summary of autophagy findings in recent literature following exercise training.

Model	Mode	Tissue	Duration of exercise, %1RM	Important Findings	Reference
Rat	Endurance Running	Cardiac Muscle	30 min 30 m min^{-1}	LC3-II ↓0h post *, ↑ 1h post*, ↑ .5h^{++}, 1h^{++}, 3h^{++} No change in p62 p-mTOR ↓ .5h^{+}, 1h^{+}	[7]
Human Young (27±2 yr) Older (70±2 yr)	Resistance Exercise	Skeletal Muscle	8 sets of 10 repetitions at 70% 1RM	Young: LC3B-II ↓ 6h and 24h post* Total protein beclin-1 ↓ in younger compared to older* Older: LC3B-II ↓ 3h*, 6h*, and 24h* post-exercise	[15]
Mouse	Endurance Running	Skeletal, Cardiac Muscle	80 min (~900 m) or maximal exercise	GFP-LC3 ↑ 0-30 min post**, 30-80*** min post-exercise	[2]
Human	Endurance Running	PBMC	60 min (70% of VO_2max)	LC3-II ↑ 2h* and 4h* post-exercise	[16]
Mouse	Endurance Running	Skeletal Muslce	4 wk training	Baseline* levels LC3-II ↑, ↓p62 in tonic oxidative muscle compared to mixed fiber types or glycolytic fibers	[1]
Human	Ultra-endurance running	Skeletal Muscle	200-km running race	LC3b* ↑ 103%, ATG4b* ↑ 49%, ATG12* ↑ 57%, Gabarap11** ↑ 286%, 3h post-race	[37]
Rat	Swim Training	Skeletal Muscle	1h x 5days, 4 weeks	↓LC3-II* exercised diabetic rats when compared to non-exercise controls	[13]
Mouse	Endurance Running	Peripheral and Brain	95 min	2-fold ↑ GFP-LC3 in cerebral cortex	[14] [9]
Mouse	Endurance Running	Skeletal Muslce	50 min, 12.3 m/min	↓ LC3-II 3h*, 6h*, 12h* post-exercise	

Notes: Values are means ± SE. 1RM = 1 repetition maximum. *Significantly different compared to rest/baseline/control ($p < .05$). **Significantly different compared to rest/baseline ($p < .01$). ***Significantly different compared to rest/baseline ($p < .001$). $^{+}$Significantly different compared to 0h post exercise ($p<.05$). $^{++}$significantly different compared to 0h post exercise ($p<.01$).

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CHAPTER III

RESEARCH MANUSCRIPT

This chapter presents a research manuscript entitled “The early time course of autophagy in human peripheral blood mononuclear cells following endurance exercise”. This manuscript is formatted for the *American Journal of Applied Physiology*. It is authored by Kathryn Rosie Lanphere, Micah Zuhl, Christine Mermier, Suzanne Schneider, Ann Gibson, Karol Dokladny, and Pope Moseley. References are provided at the end of the chapter.

The early time course of autophagy in human peripheral blood mononuclear cells following endurance exercise

Running Head: Early time course of autophagy following endurance exercise

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ABSTRACT

Exercise disrupts homeostasis and leads to the induction of an important catabolic system called autophagy. Autophagy is a beneficial cell survival process that is induced in periods of starvation and recycles cellular proteins and organelles to provide energy. The purposes of this study are to (1) determine the time course of autophagy activation following endurance exercise at 70% of VO_2max in a warm environment and (2) to determine if exercising at 50% of VO_2max in a warm environment induces autophagy. Methods. Eight endurance trained subjects (6 male, 2 female) participated in this study and completed a baseline VO_2max assessment, a moderate intensity exercise (MIE) trial for 1h (50% of VO_2max), and a high intensity exercise (HIE) trial for 1h (70% of VO_2max). HIE and MIE trials were randomized with at least 2 weeks between trials. Core temperature, heart rate, oxygen consumption, and rate of perceived exertion (RPE) were observed during exercise. Fasting peripheral blood mononuclear cells (PBMC) were collected pre-exercise, post-exercise, 30 min post-exercise, 1h post-exercise, 2h post-exercise, and 4h post-exercise. PBMCs were analyzed for mRNA and protein markers of LC3 and heat shock protein70. Plasma was analyzed for insulin and cytokine IL-6 levels using commercial ELISA kits. LC3-II, mTOR, HSP70, and Akt protein levels were measured using Western blot analysis and mRNA of LC3b and HSPA1A levels were measured by RT-PCR techniques for all time points. A two-factor repeated measures ANOVA was used to determine the effects of time and exercise intensity. A Pearson correlation analysis was used to determine the relationship between LC3-II and HSP70. Results. Data presented are mean ± SEM. Core temperature during HIE was higher during 10-60 minutes when compared to the same time during MIE and pre-HIE, $p <$

0.01. Heart rate was increased in the HIE trial from 5-60 minutes when compared to the same times during MIE and pre-HIE, $p < 0.01$. HIE and MIE did not change plasma volumes. IL-6 levels were increased, $p < 0.001$, following HIE, 2.83 ± 0.58 pg/ml, versus pre-exercise, 0.81 ± 0.28 pg/ml, and 1h post-exercise, 1.154 ± 0.26 pg/ml. IL-6 was also increased following MIE 0h post-exercise, 1.54 ± 0.26 pg/ml, when compared to pre-exercise, 0.87 ± 0.25 pg/ml, $p < 0.01$. Decreases ($p < 0.001$) in plasma insulin were found following HIE at 2h, 8.83 ± 0.60 μIU/mL, when compared to pre-exercise, 11.86 ± 1.16 μIU/mL. Decreases in plasma insulin were also found at 4h post-exercise, 9.30 ± 1.69 μIU/mL, following MIE when compared to pre-exercise, 12.14 ± 1.72 μIU/mL. Protein markers were all normalized to pre-exercise levels (1.0 ± 0.0) for the corresponding condition and protein. HIE increased ($p < 0.05$) autophagy marker LC3-II at 0h (1.37 ± 0.13), 2h (1.49 ± 0.14), and 4h (1.50 ± 0.15) post-exercise when compared to pre-exercise levels. MIE increased in LC3-II at 1h post-exercise (1.89 ±0.36) when compared to pre-exercise. The relative quantity of LC3b mRNA transcription decreased following MIE at 1h, 0.54 ± 0.09 ($p < 0.01$), and was increased at 2h, 5.00 ± 1.68 ($p < 0.05$), post-exercise when compared to baseline. No significant increases were found in HSP70 following HIE or MIE. HSPA1A mRNA, relative to β-actin, was decreased at 1h following HIE, 0.56 ± 0.10, when compared to baseline, ($p < 0.01$). HSP70 and LC3-II were moderately related during MIE, $r = 0.57$, $p < 0.001$. No significant relationship was found between HSP70 and LC3-II during HIE. Increased Akt phosphorylation occurred 2h post-MIE (1.60 ± 0.17) when compared to pre-exercise levels (1.46 ± 0.28), $p \leq 0.01$. Conclusions. For our fasted subjects, autophagy was stimulated by exercise at both 50% and 70% VO_2max. Our results suggest that autophagy is stimulated for a longer duration

following HIE. MIE induced phosphorylation of Akt post-exercise and may be activated independent of circulating insulin levels. It is unknown how or if the decreased levels we observed in plasma insulin and increased levels of IL-6 influence autophagy in PBMCs following exercise.

KEYWORDS

Autophagy; core temperature; heat-shock protein; moderate-intensity exercise; high-intensity exercise; endurance exercise

INTRODUCTION

Physical exercise is associated with cellular disturbances to homeostasis. During stress, cell signaling cascades, including the heat-shock protein (HSP) response and autophagy, maintain cell protein integrity. Exercise has recently been shown to actuate autophagy in humans and could have important health implications (13). Physiologically, endurance exercise increases circulating cytokines (20, 30), increases hypoxia and oxidative stress (31, 33), increases core temperature (1), and causes changes in insulin and glucose regulation (35), as well as muscle cell damage (30). Autophagy helps cope with these disturbances by recycling damaged cytosolic structures and by creating a source of amino acids for cells to utilize as energy for continued work (19).

HSPs are molecular chaperones that assist in the re-folding of unfolded proteins(25). A complex and coordinated relationship exists between autophagy and the heat-shock response (9). This relationship may be important in protecting cells from damage during and following exercise. Measuring autophagy and HSP expression over different time points following exercise may provide insight about possible interactions between these two systems. Findings from our lab show that there are significant increases 2h and 4h post-exercise in autophagy markers in peripheral blood mononuclear cells (PBMC) following high intensity treadmill running (70-80% VO_2max) for 1h in a warm (30˚C) environmental chamber (9). In that study it was also shown that increases in HSP70 expression, following glutamine supplementation, decreased exercise-induced autophagy (9).

In endurance exercise, the insulin-protein kinase B (Akt) pathway promotes glucose transport and glycogen synthesis in skeletal muscle (5). In this pathway, Akt is

stimulated by insulin; Akt then activates downstream targets through phosphorylation (5). Akt signaling is known to suppress autophagy via activation of mTOR (22) by regulating the phosphorylation of mTOR. In its phosphorylated state, mTOR promotes cell progress, hypertrophy, and protein synthesis(22). Due to the mTOR involvement in energy signaling, insulin and Akt may have important regulatory actions on autophagy in PBMCs. Currently, there are no studies that have examined the intensity of exercise required to increase autophagy in humans.

We investigated if moderate-intensity exercise (MIE) performed at 50% VO_2max will similarly induce autophagy and HSP70 when compared with high intensity exercise (HIE) performed at 70-80%VO_2max. The latter is known to significantly increase post-exercise markers of autophagy. We examined the early time course of autophagy markers LC3-II, HSP70, cytokine IL-6, insulin, AKT, LC3B, and HSPA1A mRNA markers in PMBCs following the two bouts of endurance exercise performed in a warm environment. The purposes of this study are to: (1) determine the time course of autophagy activation following endurance exercise at 70% of VO_2max, (2) to determine if exercising at 50% of VO_2max, (3) examine the relationship between LC3-II and HSP70, and (4) measure IL-6 and possible autophagy effectors in the insulin-Akt pathway following two bouts of endurance exercise. Our hypotheses are formulated around the 1h of endurance exercise bouts performed in a warm environmental chamber. We anticipate that: 60 minutes of endurance running at 70% of VO_2max will initiate early catabolic autophagy-mediated signaling mechanisms; 60 minutes of endurance running at 50% of VO_2max will not initiate significant autophagy signaling when compared to baseline autophagy levels; 60 minutes of endurance running at 50% of VO_2max will not

initiate significant autophagy signaling when compared to the same time points in high intensity endurance running. We also expected to find a negative relationship between LC3-II and HSP70 expression following HIE endurance running; and, there would be a negative relationship between LC3-II and HSP70 expression following MIE endurance running. Additionally, we hypothesized that IL-6 cytokine production by skeletal muscle would not have an effect on autophagy at HIE when compared to rest; IL-6 cytokine production by skeletal muscle following MIE would not have an effect on autophagy when compared to rest; decreased plasma insulin levels will activate autophagy through inhibiting the Akt signaling pathway following high intensity exercise; and, decreased plasma insulin levels will increase autophagy through inhibiting the Akt signaling pathway following moderate intensity exercise.

MATERIALS & METHODS

Subjects

This study was approved by the university's Human Research Review Committee. Healthy, endurance trained runners between the ages 18-35 were recruited from the University of New Mexico and the general Albuquerque (USA) area; they were screened for exclusion criteria. Volunteers were excluded if they self-reported to be sick, injured, dieting, had an illicit drug addiction, an inability to perform vigorous exercise, were taking antidepressant medication, diuretics, nutritional supplements that target metabolism, increase inflammation, had previously been supplementing with glutamine, or were taking any blood thinner including; Coumadin, Plavix, Pradaxa, etc. Subjects were included if they had a documented VO_2max equal to or greater than 42 ml/kg/min (men) or greater than or equal to 38 ml/kg/min (women), or if they were able to meet the

VO_2max criteria following baseline assessment on their first visit to the lab. Ten subjects (8 men) satisfied all criteria and completed an informed consent, HIPAA consent, health history questionnaire, and medical history form screening. All testing was performed in the exercise laboratory at the University of New Mexico at an altitude of 1524 m.

Experimental Design

The study utilized a repeated measures design. Each subject participated in 2 exercise trials separated by no less than a 2-week wash-out period in which subject's maintained normal dietary and exercise routines. During each exercise trial, the subjects exercised on a treadmill for 1h in a warm (30˚C) environmental chamber. Exercise trial sequence was randomized by the flip of a coin and consisted of the high intensity exercise (HIE) trial set at 70% of VO_2max and the moderate intensity exercise (MIE) trial set at 50% of VO_2max. On the first visit to the lab, baseline measurements (maximal oxygen consumption, height, weight, age, and 3 site skinfolds) were performed on each subject. One week later subjects returned to the lab after an overnight fast to complete the first exercise trial. Subjects continuously exercised for 1h as long as a core temperature of 40˚C was not exceeded. Venous blood samples were taken to determine the autophagy time course, HSP70, IL-6, insulin levels, and Akt post exercise, 30 minutes post, 1 hour post, 2 hours, and 4 hours post exercise from the antecubital vein (Figure 1).

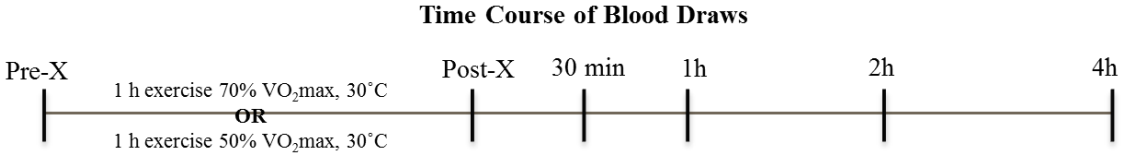

Figure 1. Time course of blood draws diagram. Time of blood draws are labeled (Pre-X) pre-exercise, (Post-X) post-exercise, (30 min) 30 minutes post exercise, (1h) 1 hour post exercise, (2h) 2 hours post exercise, (4h) 4 hours post exercise.

Baseline Measurements

Body mass (to the nearest kg) was measured on a physician's balance-beam scale, while the participants were without shoes and wearing running attire. Height (to the nearest 0.5 cm) was measured with the subjects standing upright, without shoes, at a wall mounted scale. Body density was calculated by skinfold technique according to the three site skinfold methods of Jackson and Pollock (men: chest, abdominal, thigh, women: triceps, suprilliac, thigh), Equation 1 and 2, and fat free mass was calculated following the conversion of body density to body fat percentage using the Siri equation, Equation 3.

Equation 1. Body density for men: (15)

$$Body\ Density\ \left(\frac{g}{ml}\right) = 1.10938 - \left(0.0008267\ \times \sum chest, abdomen, thigh\right) + (0.0000016 \times \left(\sum chest, abdomen, thigh^{2}\right) - (0.0002574 \times age)$$

Equation 2. Body density for women:(16) $Body\ Density\ \left(\frac{g}{ml}\right) = 1.0994921 - (0.0009929 \times \sum triceps, thigh, suprailiac) + (0.0000023 \times (\sum triceps, thigh, suprailiac)^{2}$

Equation 3. Siri equation for determining body fat percentage:(34)

$$Body\ fat\ \% = \left(\frac{495}{Body\ Density}\right) - 450$$

Maximal aerobic capacity (VO_2max) was measured using open circuit spirometry (Parvomedics, Sandy, UT, USA) during a continuous incremental protocol on a motorized treadmill. The highest value for oxygen consumption based on an 11-breath running average was used to determine the subject's VO_2max (2). At least 3 of the following criteria were met to confirmVO_2max: plateau of oxygen consumption (<150 ml/kg/min), RER (>1.15), RPE (>17), and heart rate (± 10 bpm of age-predicted maximal

heart rate). A heart rate monitor (Polar Electro Inc., Lake Success, NY, USA) was used to measure subject's heart rates.

Generalities of blood sampling

Using antiseptic technique, blood samples were drawn to determine the autophagy time course, HSP70, IL-6, insulin level, Akt, LCB, and HSPA1A mRNA at pre- and several time-points post-exercise (Figure 1). Prior to each blood draw, subjects were seated for 10 min to control for shifts in plasma volume due to posture. Venous blood (20 ml each draw) was collected post-exercise, 30 min post, 1h post, 2h post, and 4h post-exercise (120 ml of total blood drawn). Blood was drawn into EDTA tubes for short-term storage and transportation. Blood was also drawn into microhematocrit tubes. At this time, the microhematocrit tubes were centrifuged for analysis of hematocrit and plasma volume. Microhematocrit tubes were then centrifuged for 10 minutes at 13,000 rpm. Hematocrit was measured in triplicate and the two most similar values were averaged and used for analysis.

Calculation of percentage changes in volumes of plasma and red cells was determined from blood drawn at each time point. Hematocrit was corrected for plasma trapped with the packed red cells in accordance with the technique of Dill and Costill (7). Corrected hematocrit values were then used to determine the percentage of plasma volume change (supplemental Figure 11) in accordance with the technique of van Beaumont (Equation 4) where Hct indicates hematocrit, pr indicates sample prior to exercise, and po indicates sample post-exercise (36).

Equation 4. Percent plasma volume change: (36)

$$\%\Delta Plasma\ Volume = \frac{100}{100-Hctpr} x\ \frac{Hctpr-Hctpo}{Hctpo}\ x\ 100$$

Whole blood (20 ml) collected into EDTA tubes was transported to our lab at the university's Clinical and Translational Science Center, gently laid onto 20 ml of histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 2,400 rpm for 30 min at 20˚C (29). Two 1.5 ml Eppendorf tubes of plasma were immediately frozen at -80˚C. The remainder of the plasma was pipetted and discarded. The PBMC layer was pipetted into 10 ml of phosphate buffered saline (PBS, Sigma Aldrich 4417, St. Louis, MO) and centrifuged at 2400rpm for 10 min at room temperature. PBS was discarded and cells were washed two more times with PBS. Cells were counted under a high powered microscope and four million cells were aliquoted into Eppendorf tubes and frozen at -80°C for later analysis.

Exercise protocol

All subjects participated in two exercise trials in which they exercised at a constant exercise intensity of 50% of their VO_2max (MIE) and at 70% of VO_2max (HIE). Subjects reported to the lab one week following baseline testing to complete their first exercise trial. On exercise trial days, subjects arrived at the laboratory after an overnight fast. Weight was recorded and the subjects were fitted with a Polar heart rate monitor. Subjects inserted a thermistor 13 cm into the rectum for core temperature monitoring. Subjects exercised at the designated intensity at 30˚C for 1h or until a core temperature of 40˚C was reached. Subjects were instructed to drink water *ad libitum* but remained fasted for the duration of the day's session. Heart rate (HR), core temperature, and the rating of perceived exertion (RPE) were recorded every 5 minutes during exercise. A PARVO metabolic system was calibrated inside the environmental chamber and used to monitor and record oxygen consumption every 10 minutes during exercise trials;

workload was adjusted as appropriate. Two weeks later, the subjects repeated the protocol but at the other intensity.

Protein extraction and quantification

PBMCs were homogenized by placing them on ice for 25 minutes with 250 µl of RIPA commercial lysis buffer and then the lysate was centrifuged for 10 minutes at 4˚C (27). The protein-containing supernatant was collected and stored in 1.5 ml Eppendorf tubes for protein quantification. Protein quantification was performed using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). A 4X Laemmli buffer with 5% β-mercaptoethanol was added to the protein lysate and incubated at 100˚C for 10 minutes before gel loading for protein separation.

Western blot protein analysis

Proteins were separated by electrophoresis on a 12% resolving and 6% stacking sodium dodecyl sulfate polyacrylamide gel. A 15 µg volume of protein was loaded onto gels. Separated proteins were transferred to a trans-blot transfer nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes (12%) were blocked for 1 hour in 5% dry milk and Tris-buffered saline Tween 20 buffer solution, washed in Tris-buffered saline (TBS), and incubated overnight in the corresponding primary antibody (LC3-II and HSP70) at 4˚C (23). Membranes (6%) blotted for p-Akt and p-mTOR were blocked for 1 hour in Membrane Blocking Solution (Invitrogen, Carlsbad, CA) containing 0.1% proclin. Following measurement of p-Akt and p-mTOR, membranes were stripped and re-probed with their corresponding antibodies for total Akt and total mTOR protein. All membranes were washed in TBS with 0.05% Tween 20 (TBS-tween) and incubated with a horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA) and

incubated for 1 hour at room temperature. Santa Cruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA) and Kodak BioMax MS film (Fisher Scientific, Pittsburg, PA) were used to develop the membrane. The following primary antibodies and relative dilutions were used: rabbit polyclonal anti-LC3-II (Sigma-Aldrich), rabbit polyclonal anit-HSP70 (Cell Signaling, Danvers, MA), rabbit polyclonal anti-mTOR (Cell Signaling), rabbit polyclonal anti-Akt (Cell Signaling), rabbit polyclonal anti-P-mTOR (Cell Signaling), rabbit polyclonal anti-P-Akt (Cell Signaling), mouse polyclonal anti-β-actin (Invitrogen). Adobe Photoshop (San Jose, CA) was used to quantify protein expression by determining densitometric values. HSP70 and LC3-II were standardized to the endogenous housekeeping protein β-actin (supplemental Figure 12).

ELISA

An enzyme linked immunosorbant assay (ELISA) kit was used (Quantikine HS ELISA, R&D Systems, Minneapolis, MN) to determine the concentration of human interleukin-6 (Il-6), sensitivity of 0.11 pg/mL, and insulin, sensitivity of 1.76 IU/mL, (Insulin ELISA, DRG Instruments GmbH, Germany) according to the manufacturer's instructions. IL-6 and insulin concentrations were measured in plasma pre-exercise, post-exercise, 1, 2, and 4 hours post-exercise. IL-6 and insulin concentrations were corrected for changes in plasma volume following exercise (37). A four parameter logistic fit (MyAssays Ltd Software) was used to calculate IL-6 and insulin concentrations from optical density measurement and the standard curve ($R^2 = .9998$) as suggested by the manufacturer (supplemental Figure 13).

Isolation of total RNA from PBMCs

Total cellular RNA was isolated from the PBMCs. A QIA-RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) was used to disrupt cells, isolate, and purify total RNA content per the manufacturer's protocol. Total RNA content was then measured and checked for purification by a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

Reverse transcription for RNA detection

Reverse transcription was performed to create complementary DNA (cDNA) from single stranded RNA using the Transcription First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). One reaction for cDNA synthesis consisted of 4 µl of a 5X RT buffer A (PE Biosystems); 4.1 µl Rnase free water; 2 µl dNTPs, 2 µl RdHx (50 pmol/µl), 0.5 µl RNase inhibitor (40 U/µl), 0.4µl RT enzyme, and 7 µl RNA. The reactions were incubated at 65˚C for 5 min and then at 25˚C for 10 min followed by a 60-minute incubation at 20˚C, and 85˚C for 5 minutes to denature the RNA secondary structure. Following creation of cDNA each sample was checked for concentration and purity with the NanoDrop 1000. The cDNA samples were aliquoted and stored at -20˚C.

Quantitative Real-time PCR: TaqMan detection

Human HSP70 (HSPAIA, Applied Biosystems, Foster City, CA) and LC3-B (MAP1LC3, Applied Biosystems) messenger RNA was quantified using real-time PCR. Taqman master mixes and probes were used according to the manufacturer's recommendations (Applied Biosystems). All reactions were performed in duplicate on a MicroAmp 96-well plate (Applied Biosystems) capped with Micro-Amp optical caps, normalized to the endogenous control β-actin, and HIE pre-exercise samples (32).

Statistical Analysis

A power analysis was performed *apriori* to determine the appropriate sample size for a power of 0.8 and an alpha level of 0.5 (effect size = .456). G*Power 3.1.3 statistical software was used and generated a sample size of 8 subjects. (11) Statistical analyses were performed using GraphPad Prism, Version 3.02 (La Jolla, CA, USA) and SPSS Statistics 17.0 (Chicago, IL). Prior to statistical analysis, all proteins analyzed by Western-blot technique were normalized to total protein content (for phosphorylated proteins) or β-actin. In order to compare multiple blots, protein quantity was also normalized to the pre-exercise protein level that corresponded with the matched exercise intensity. A 2-factor repeated measures ANOVA was used to determine the effects and interactions of exercise intensity (HIE, MIE) and time (pre, 0h, .5h, 1h, 2h, and 4h) for core temperature, HR, LC3-II, HSP70, IL-6, insulin, mTOR, and Akt proteins. Paired *t*-tests were used to determine differences in mRNA HSPA1A and LC3b due to missing data points. A Pearson correlation analysis was used to determine the relationship between HSP70 and LC3-II following exercise. If statistically significant interactions were found, LSD *post hoc* testing was used to determine differences in sample means.

RESULTS

Subject physiological characteristics

Eight subjects (6 male, 2 female) completed a VO_2max assessment, and both MIE and HIE bouts. A total of 10 subjects were recruited for this study. Two subjects chose to withdraw from the study and their data was withdrawn from analyses. Subjects (Table 1) were trained runners and were able to complete both exercise trials at the designated intensities without incident. There were no differences in the age, height, body mass, body density, or VO_2max between male and female participants.

Table 1. Subject Characteristics.

	Age (years)	**Height (cm)**	**Body Mass (kg)**	**VO_2max (ml/kg/min)**	**Body Fat %**	**BMI (kg/m^2)**	**Body Density (g/ml)**
Males (n=6)	27.2 ± 1.7	181.6 ± 4.6	82.7 ± 3.0	50.0 ± 2.1	11.6 ± 4.9	25.1 ± 0.5	1.07 ± 0.004
Females (n=2)	29.0 ± 0.0	172.8 ± 6.3	71.5 ± 8.3	46.2 ± 5.1	25.1 ± 4.9	23.9 ± 1.1	1.04 ± 0.007

Data are mean ± SEM

Core temperature

Significant main and interaction effects were found for time ($F(12,84)=60.946$, $p<.001$), exercise intensity ($F(1,7)=22.395$, $p<.01$), and the interaction of time and exercise intensity ($F(12,84)=13.567$, $p<.001$). LSD *post hoc* testing showed temperature was significantly higher during HIE at10 min ($p<.05$), 15 min ($p=.01$), and for each subsequent 5-minute increment, $p<0.001$, when compared to the same time points during MIE (Figure 2).

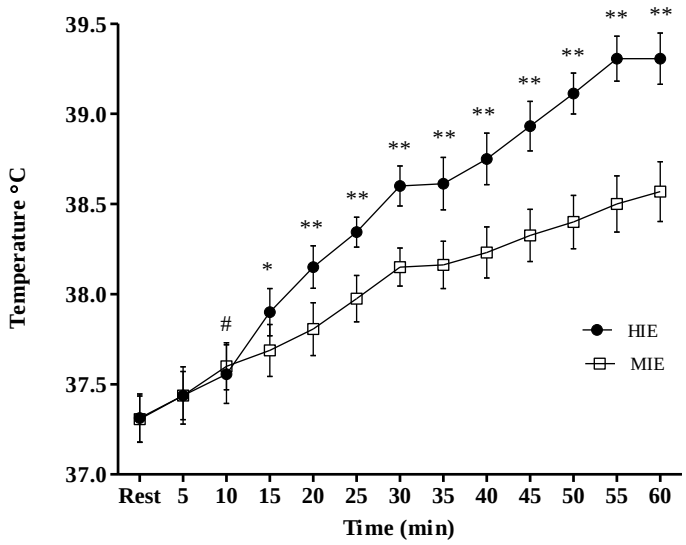

Figure 2. Core temperature responses during HIE. Core temperature was significantly higher during 10 min through 60 min of HIE when compared to the same time points during MIE. Data are mean ± SEM, N = 8. #p < 0.05, *p=0.01, and **p<0.001.

Heart rate

The effects of intensity, time, and the interaction between time and intensity were all statistically significant (F(1,7)=64.331, p<.001; F(12,84)=37.041, p<.001; F(12,84)=3.066, p=.001, respectively). LSD *post hoc* testing showed increased heart rate following 5 minutes of HIE and each 5 min increment to 60 min of exercise, p<.001, when compared to MIE (Figure 3).

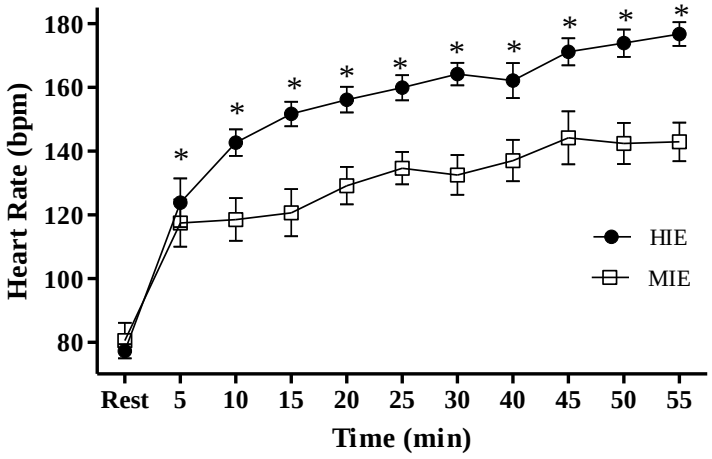

Figure 3. Heart rate responses during HIE and MIE. Heart rate was significantly higher at each 5 min interval during HIE when compared to MIE. Data are mean ± SEM, N = 8. *p<0.001

Plasma volumes

Measurement of plasma volume was posture controlled and corrected for trapping of plasma in the red cell content (7). There were no differences in the plasma volume percentage between MIE and HIE or at any time points pre- or post- exercise (Supplemental Figure 1).

Cytokine IL-6 concentration

IL-6 cytokine concentrations were corrected for changes in plasma volume according to van Beaumont et al. (37). The effect of time was statistically significant ($F(4,28) = 11.38$, $p < 0.001$). There were no statistically significant effects of exercise intensity or interactions between time and intensity on IL-6 cytokine concentration. Table 2 presents means ± SEM for cytokine data.

Table 2. IL-6 and insulin following 1h of exercise at high intensity endurance exercise (HIE) and moderate intensity endurance exercise (MIE). Time following exercise is shown as is the associated p-value.

Intensity (% of VO_2max)	Time (h)	IL-6 (pg/ml)	p-value	Insulin (µIU/mL)	p-value
HIE (70 %)	Pre	0.81 ± 0.28	-	11.86 ± 1.16	-
	0	2.83 ± 0.58*	< 0.01	17.51 ± 3.36	0.07
	1	1.54 ± 0.26*	< 0.01	11.48 ± 1.18	0.10
	2	1.30 ± 0.23	0.11	8.83 ± 0.60	0.03
	4	1.45 ± 0.18	0.06	10.85 ± 1.61	0.17
MIE (50%)	Pre	0.87 ± 0.25	-	12.14 ± 1.72	-
	0	1.54 ± 0.28*	< 0.01	14.85 ± 2.56	0.17
	1	1.10±0.17	0.29	11.23 ± 2.09	0.39
	2	1.02±0.30	0.65	11.60 ± 1.74	0.82
	4	0.90±0.21	0.94	9.30 ± 1.69	0.03

Data are mean ± SEM, N=8. * Statistically different than pre-exercise within the intensity.

Post hoc testing using LSD showed IL-6 levels significantly ($p<0.001$) higher post-exercise (0h), and 1h post-exercise, during HIE when compared to pre-exercise (Pre). During MIE, post-exercise (0h) IL-6 levels were found to be increased, from pre-exercise (Figure 4).

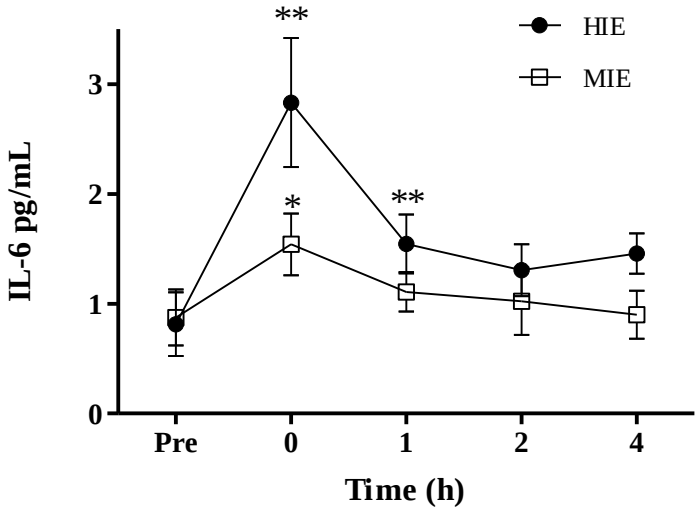

Figure 4. Plasma IL-6 cytokine concentrations during HIE and MIE. When compared to pre-exercise, post-exercise (0h) and 1h post-exercise cytokine levels are increased following HIE. Following MIE, IL-6 was elevated post-exercise (0h) when compared with pre-exercise. Data are mean ± SEM, N = 8. *p<.01, **p<.001.

Insulin

Insulin concentrations were corrected for changes in plasma volume according to van Beaumont et al.(37). Time had a significant effect ($F(4,28) = 7.718$, $p < 0.01$) on insulin concentrations. No interaction was also found between intensity and time. LSD *post hoc* testing showed decreases in insulin following HIE 2h post-exercise ($p < 0.05$) when compared to pre-exercise insulin levels. During MIE, insulin was decreased 4h post-exercise when compared to pre-exercise, $p < 0.05$ (Figure 5).

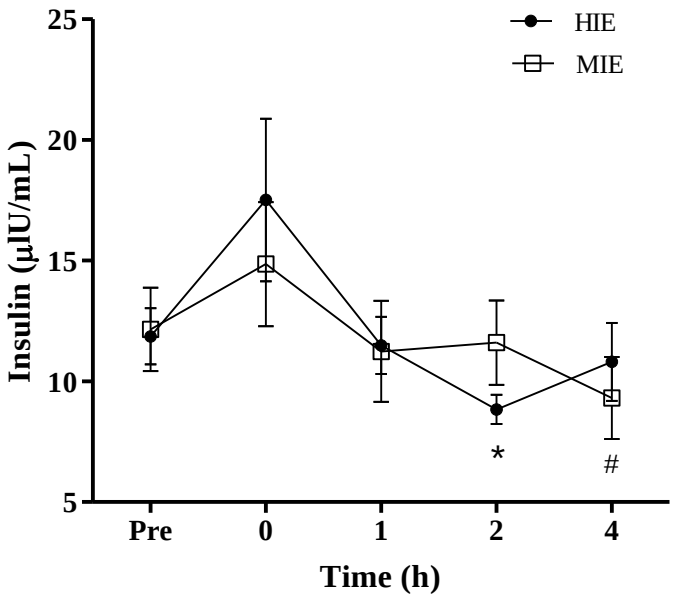

Figure 5. Plasma insulin concentrations following HIE and MIE. When compared to pre-exercise, insulin levels significantly decrease 2h following HIE. Following MIE, insulin were decreased 4h post-exercise when compared with pre-exercise. Insulin concentrations were adjusted for changes in plasma volume. Data are mean ± SEM, N = 8. Statistically decreased from pre-exercise *p<.05 in HIE compared to Pre, $^{\#}p < 0.05$ in MIE compared to Pre.

Protein kinase B (Akt)

Means ± SEM values are shown in Table 3. There were no significant effects or for or interactions between time and intensity. Paired *t*-tests were used to determine the differences within exercise intensities. Akt was increased 2h following MIE when compared to pre-exercise ($p < 0.01$). Following MIE, Akt was decreased, $p < 0.01$, 1h and 4h post-exercise when compared with 2h. No significant changes were observed following HIE (Figure 6).

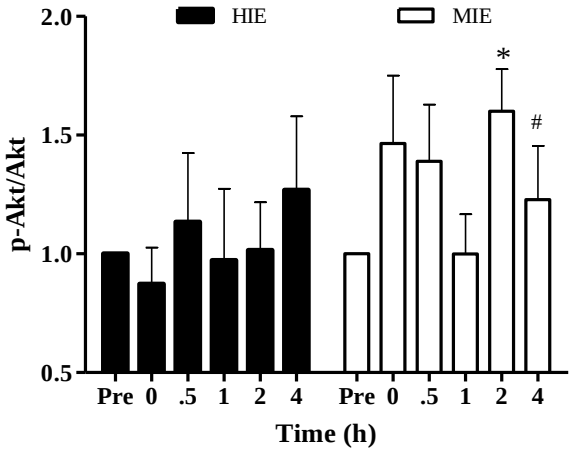

Figure 6. Protein kinase B (Akt) following HIE and MIE. Increased levels of Akt protein were found 2h post-exercise following MIE when compared to pre-exercise and 1h post-exercise in the same intensity. No differences were found following HIE. Within MIE intensity, Akt was also found to be significantly decreased 4h post-exercise when compared to 2h post-exercise. Significantly different than pre-exercise and 1h, *$p \leq 0.01$, significantly different than 2h, $^{\#}p < 0.01$

Autophagy marker LC3-II protein

LC3-II was normalized to β-actin; the ratio mean ± SEM and p-values compared to pre-exercise values are presented in Table 3. HIE increased LC3-II protein 0h, 2h, and 4h post-exercise when compared to pre-exercise (Figure 7). An increase in LC3-II ($p < 0.05$) was also observed 1h post-exercise following MIE when compared to pre-exercise. Time was a significant main effect ($F(5,40) = 3.661$, $p < .01$). No statistically significant interactions or intensity effects were found. A one-way repeated measures test was also performed to determine the change of LC3-II with time for each exercise intensity. A statistically significant interaction between means was found following HIE, $p = 0.04$. No interactions were found during MIE, $p = 0.22$.

Table 3. LC3-II/β-actin, HSP70/β-actin, and p-Akt/Akt ratios following high intensity endurance exercise (HIE) and moderate intensity endurance exercise (MIE).

Intensity (% of VO_2max)	Time (h)	LC3-II/β-actin (units)	p-value	HSP70/β-actin (units)	p-value	p-Akt/Akt (units)	p-value
HIE (70%)	0	1.37 ± 0.13*	0.02	1.62 ± 0.12	0.12	0.87 ± 0.15	0.43
	.5	1.12 ± 0.17	0.51	1.73 ± 0.36	0.19	1.13± 0.29	0.65
	1	1.31 ± 0.17	0.11	1.72 ± 0.51	0.11	0.97 ±0.30	0.93
	2	1.49 ± 0.14*	0.01	1.51 ± 0.39	0.23	1.01 ±0.20	0.94
	4	1.50 ± 0.15*	0.01	1.28 ± 0.44	0.54	1.26 ±0.31	0.41
MIE (50%)	0	1.4 ± 0.23	0.12	1.29 ± 0.22	0.29	1.46±0.28	0.14
	.5	1.33 ± 0.20	0.15	1.23 ± 0.18	0.22	1.39±0.23	0.14
	1	1.89 ±0.36*	0.04	1.52 ± .26	0.08	1.0±0.16	0.99
	2	1.98 ± 0.56	0.12	1.12 ± 0.06	0.11	1.60±0.17*	0.01
	4	1.57 ± 0.44	0.23	1.48 ± 0.25	0.09	1.22±0.22	0.34

Data are mean ± SEM, N=8. * Statistically different than pre-exercise within intensity.

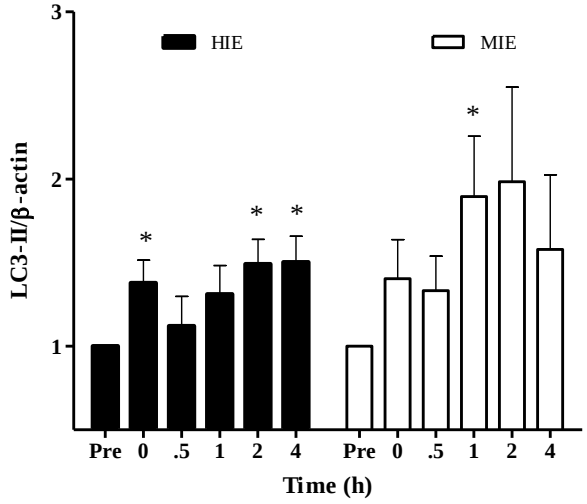

Figure 7. Normalized LC3-II protein during HIE and MIE protocols. LC3-II was increased following MIE 1h post-exercise when compared to pre-exercise. HIE increased LC3-II protein expression post-exercise (0h), 2h, and 4h when compared to pre-exercise. Data are mean ± SEM, N=8. *p<0.05.

Q-RT PCR analysis of LC3b

The relative quantity of LC3b, normalized to pre-exercise and compared to β-actin, are shown in Table 4 as means ± SEM. Paired *t*-tests were used to determine the differences in LC3b within exercise intensities due to missing data points. No differences were found following HIE (Figure 8). A decrease in LC3b was found 1h post-MIE when compared to pre-MIE, $p<0.01$. An increase in LC3b was found 2h post-MIE when compared to pre-MIE, $p<0.05$.

Table 4. Q-RT PCR analysis of LC3b and HSP70 following HIE and MIE. Data shown are comparative (ΔCT) relative quantities (RQ) adjusted to Pre condition cycle thresholds and endogenous control β-actin.

Intensity (% of VO_2max)	**Time (h)**	**LC3b (RQ)**	**p-value**	**HSPA1A (RQ)**	**p-value**
HIE (70%)	Pre	1.0 ± 0.0	-	1.0 ± 0.0	-
	0	0.9 ± 0.38	0.41	5.14 ± 2.05	0.08
	.5	1.43 ± 0.46	0.30	6.74 ± 5.07	0.29
	1	1.40 ± 0.78	0.30	11.02 ±6.57	0.17
	2	1.66 ± 0.69	0.33	11.17 ±8.99	0.29
	4	1.36 ± 0.72	0.34	16.17 ± 11.74	0.24
MIE (50%)	Pre	1.0 0.0	-	1.0 ± 0.0	-
	0	0.55 ± 0.21	0.08	0.72 ± 0.24	0.30
	.5	4.836 ± 2.54	0.17	6.25 ± 4.07	0.24
	1	0.54 ± 0.09*	< 0.01	0.56 ± 0.10*	< 0.01
	2	5.00 ± 1.68*	< 0.05	4.49 ± 1.79	0.09
	4	1.40 ± 0.36	0.31	1.55 ±0.46	0.26

Data are mean ± SEM, N=8. * Statistically different than pre-exercise within intensity.

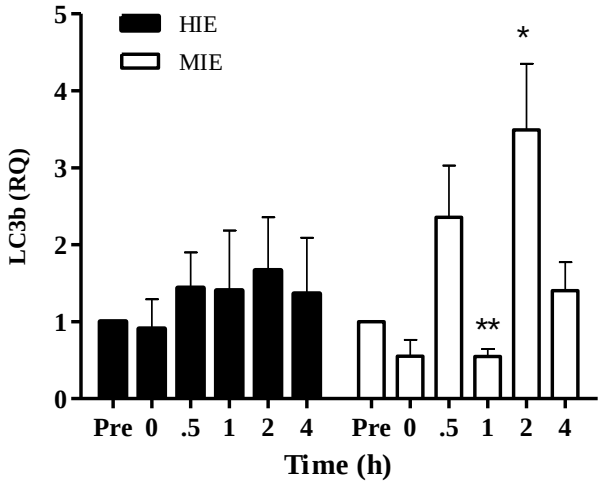

Figure 8. Relative quantity (RQ) of LC3b mRNA following HIE and MIE. No statistical differences were found following HIE. A decrease in LC3b occurred 1h post-MIE. LC3b was increased 2h post MIE when compared to pre-exercise levels. Data are mean ± SEM, N=8. **p < .01, * p < 0.05.

Heat shock protein 70

There were no significant effects of time, exercise intensity, or interactions between factors (Figure 9). Table 3 presents the mean ± SEM for HSP70 protein normalized to β-actin for each time point. No differences were found, however, a trend for increasing HSP70 is observed following exercise at both intensities.

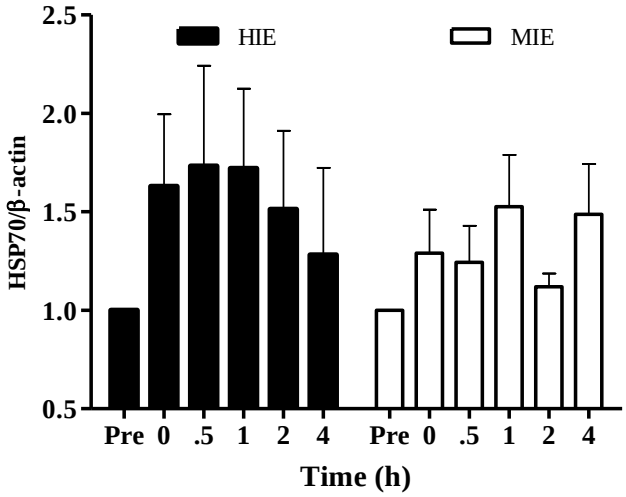

Figure 9. Trend for HSP70 in PBMCs following HIE and MIE. No statistically important differences were found within or between groups.

Q-RT PCR analysis of HSPAIA

The relative quantity of HSPA1A, normalized to pre-exercise and compared to β-actin, are shown in Table 4 as means ± SEM values. Paired *t*-tests were used to determine the differences in HSPA1A within exercise intensities due to missing data points as a result of instrument error. No differences were found following HIE (Figure 10). A significant decrease in HSPA1A was found 2h post-MIE when compared to pre-MIE, $p<0.01$.

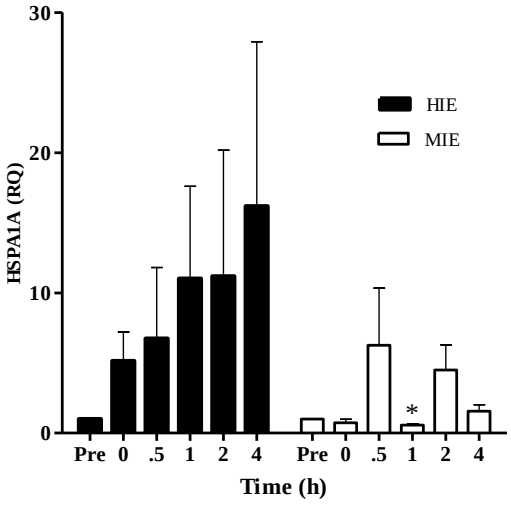

Figure 10. Relative quantity (RQ) of HSPA1A mRNA following HIE and MIE. An increasing trend was found for HSPA1A following HIE. No statistical differences were found following HIE. HSPAIA was decreased at 1h post-MIE ($p < 0.01$). Data are mean ± SEM, N=8.

Relationship between heat shock protein 70 and LC3-II

To determine the relationship between HSP70 and LC3-II a correlation was performed (Figure 11). A moderately strong positive relationship was found for MIE ($r = 0.576$, $p < .001$). No significant relationship was found for HIE.

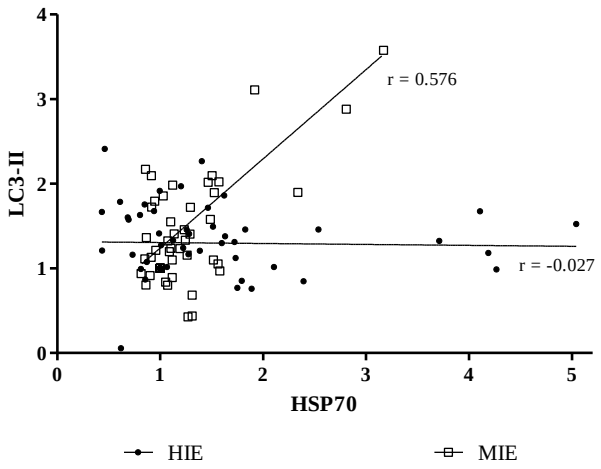

Figure 11. Correlations between HSP70 and LC3-II during HIE and MIE. Correlation analysis of HSP70 and LC3-II protein during two exercise intensities revealed a moderate correlation between HSP70 and LC3-II during MIE, $p < 0.001$. There was a slightly inverse and weak relationship between HSP70 and LC3-II, $p = 0.843$.

DISCUSSION

Physiological changes occur in response to cell signaling transduction networks following skeletal muscle contraction in exercise. The stress of high-intensity exercise (HIE) increases autophagy however; the mechanisms by which exercise stimulates autophagy have not been previously designated. The aim of this study was to determine the early time course of exercise-induced autophagy markers in PBMCs following physiological changes caused by contractile activity of endurance exercise at two intensities. Additionally, we wanted to determine the relationship between autophagy marker LC3-II and HSP70 following exercise. We targeted the insulin-insulin-like growth factor signaling pathway due to its roles in regulating the glucose transport that is needed to sustain endurance exercise (5). In agreement with previous findings published by our lab, we show that autophagy can be stimulated in PBMCs by exercise performed

in a warm environment and at a high-intensity (9). Additionally, we now show that moderate-intensity endurance exercise can also stimulate autophagy in PBMCs.

Regular exercise is associated with protection against premature death due to disease. This protection has been attributed to the anti-inflammatory effects of exercise practiced regularly since regular exercise induces endocrine effects that mediate the anti-inflammatory response of cytokines (4). Cytokines are known modulators of autophagy and can be stimulated by strenuous exercise, stress hormones, energy crisis and oxidative stress (30). In our current study, acute increases of cytokine IL-6 following 1h of endurance exercise at both intensities was reported. HIE resulted in an average increase in IL-6 approximately of 2.0 pg/ml from pre- to post-exercise. By post-exercise, IL-6 levels were falling yet remaining increased when compared to pre-exercise levels. Normal circulating IL-6 in humans is in the 1 pg/mL range (38) which is similar to the fasted level of IL-6 we observed before exercise. IL-6 was also nearly doubled immediately post-exercise with MIE when compared to pre-exercise. Another study also investigated exercise intensity effects on anti-inflammatory cytokines. Peake et al. (30) compared the effects of exercise intensity and muscle damage on changes in anti-inflammatory plasma cytokines in moderate-intensity treadmill running (60% VO_2max), high-intensity treadmill running (85% VO_2max), and downhill running (60% VO_2max). These authors found that, following exercise that lasted up to 1h in duration, the intensity appeared to have a greater effect on anti-inflammatory cytokine production than on exercise-induced muscle damage (30). Cytokines were also found to be most increased following high-intensity running (30).

It is thought that increases in cytokine levels may serve to inhibit autophagy. A study investigated the role of cytokines IL-4 and IL-13 on macrophages and IFN-ϒ induced autophagy (12). Harris et al.(12) found that IL-4 and IL-13 cytokines inhibited IFN-ϒ-induced autophagy and was independent of the Akt pathway. In the same study, they also found that IL-4 and IL-13 also inhibited starvation-induced autophagy in a manner dependent on Akt signaling (12). Dutta et al.(10) found that IL-6 also interfered with IFN-ϒ signaling and inhibited starvation induced autophagy. Mechanistically, IL-6 was found to dependently lower the Atg12-Atg5 complex and inhibit autophagosome biogenesis (10). We have shown an association between reduced autophagy following MIE and increases in IL-6 but the explicit role that circulating IL-6 has on autophagy inhibition in PBMCs is not yet known and may be intensity-dependent.

The secretion of insulin is controlled by plasma glucose concentrations and is the principal hormone responsible for glucose metabolism. A previous study has shown that acute aerobic exercise is known to increase insulin following 45 min of high-intensity cycling (alternating 2 min at 25% and 2 min at 90% of VO_2peak), 37.7±22.7μU/ml, and 60 min following moderate-intensity cycling (50% of VO_2peak), 30.7 ± 17.6μU/ml, in fed individuals when compared to fasted pre-exercise measures, 6.9 ± 2.1 and 8.0 ± 3.2μU/ml, respectively (35). Their study also showed that insulin dramatically decreased 2h post-exercise, compared to baseline, and returned to below resting levels 6h after exercise (35). We measured insulin levels in fasted individuals at baseline and 4 time points following endurance exercise. Our study showed similar trends to data published by Trombold et al., however, our subjects were fasted prior to exercise. Pre-HIE insulin levels were 11.86 ± 1.16 μIU/mL and then significantly decreased below baseline levels

2h post-exercise to 8.83 ± 0.60 μIU/mL. The MIE protocol also resulted in a significant decrease from baseline insulin (12.14 ± 1.72 μIU/ml), at the 4h post-exercise time point (9.30 ± 1.69 μIU/ml). The response we observed was expected considering the greater glucose demands of contractile muscle, in addition to the greater demand and need to replace muscle glycogen following HIE versus MIE. The decrease in insulin following MIE occurred later than the insulin decrease following the HIE protocol. This decrease below baseline levels is thought to inhibit the insulin-insulin like pathway and, in theory, activate autophagy auto-induction.

Protein kinase B (Akt) is proposed to regulate signaling responses, such as phosphorylation of mTOR. Cell progress, hypertrophy, and protein synthesis are promoted by phosphorylated mTOR. In this pathway, Akt is stimulated by insulin and activates downstream targets by phosphorylation (5). Akt signaling is known to suppress autophagy via activation of mTOR (22). Recent findings indicate that endurance exercise results in phosphorylation of Akt (p-Akt) which promotes glucose transport and glycogen synthesis in skeletal muscle (5). Peak phosphorylation for many Akt-related signaling proteins was found to occur around 30-60 min after cycling exercise for 1h at 70% of VO_2max in the fasted state (5). We also showed an increase in p-Akt, the active protein, in PBMCs 1h post-MIE. During the MIE trial, HR and core temperatures were statistically lower than during the HIE trial. The decreased physiological stress associated with the MIE trial in the same warm environment may have allowed cell signaling progression, through the phosphorylation of mTOR, to occur and, henceforth, increase p-Akt as we observed 1h post-exercise. We found no increases in p-Akt following HIE. Our data showed decreases in plasma insulin following HIE and the downstream p-Akt was

also not detected. This null observation of p-Akt following HIE, in theory, would have precluded the phosphorylation of mTOR thereby promoting increased autophagy. Camera et al. (5) reported that coordinated changes of p-Akt in muscle tissue occurred independent of blood glucose or insulin concentrations. Other human studies have reported increases, decreases, or unchanged responses (3, 6) of Akt signaling in muscle cells following endurance exercise (5). These inconsistencies may have been due to differences in exercise intensity, duration, subject training status, or timing of the tissue collection. Despite differences in tissue used for our analysis, the role of Akt in PBMCs is assumed to be similar to other tissues and involved with glucose transport mechanisms and cell progression activities (5).

Perhaps the most novel finding in the present study is that autophagy can be stimulated by both moderate- and high- intensity exercise performed in a warm environment. One aim of this study was to determine if exercise at a moderate-intensity could stimulate autophagy as shown in higher-intensity exercise studies (9). We showed that autophagy is significantly increased 1h post-moderate-intensity treadmill running or brisk walking. The stress of HIE in a warm environment was also shown to induce autophagy at several time points following exercise. Specifically, HIE induced LC3-II protein 0h, 2h, and 4h post-exercise. Exercise intensity was not a significant factor in LC3-II expression; both HIE and MIE were sufficient to stimulate autophagy. In catabolic situations such as endurance exercise, a coordinated system is needed to supply alternative energetic substrates for working muscle and cells. Autophagy is thought to provide this needed energy substrate. In rat cardiac muscle, a single bout of running exercise for 30 min resulted in a “biphasic” change in autophagy with an initial decrease

observed immediately after exercise and a subsequent increase 1 h thereafter (28). We observed this "biphasic" effect of LC3-II flux in PBMCs following both HIE and MIE. By investigating the time course of autophagy we were able to observe these phases in two intensities in order to help identify the optimal conditions and times to induce autophagy in future studies. The time course and regulation of autophagy by the Akt pathway is complex and may occur independent of insulin. Time following exercise in fasted individuals was found to have the greatest effect of LC3-II translation. It is important that the time of tissue collection for future studies be coordinated by the autophagy time course in order to determine responses to the intervention.

Upregulation of autophagy-related genes have been seen following ultra-endurance exercise in skeletal muscle (17). Jamart et al. showed that following a 200 km race, LC3b mRNA was increased by 103% 3h following an ultramarathon (17). We showed a decrease in LC3b mRNA at 1h post-MIE and an increase 2h post-exercise when compared to baseline levels. We also did not show an increase in LC3b following HIE. When autophagy is induced, the membrane bound protein form of LC3-II is converted from the soluble protein form LC3-I by several autophagy related genes, such as PE, Atg3, Atg4, and Atg7 (18). The conversion of LC3-I to LC3-II is ultimately required for membrane expansion and is a good indicator of autophagosome formation (18, 24). Since LC3-I is already present inside the cytosol, the transcription of LC3b mRNA may not be necessary for LC3-II to be detected. Exercise has been shown to decrease autophagy marker LC3-II at 3, 6, and 12h post-exercise in murine skeletal muscle following 50 min of running elsewhere. However, the mice were provided with chow and water *ad libitum* (18). Swim training studies on diabetic rats have also reported

decreased baseline levels of autophagy and increased muscle mass when compared to diabetic non-exercised controls (21). Lee et al. (21) attributed the loss of muscle mass in diabetic rats to hypercatabolic metabolism associated with autophagy. Although our protein data do not support these studies, the feeding state, altered health and physiological state of the animals, non-human models used for analysis, and time point at which autophagy was measured should be taken into consideration. In other rat models, treadmill exercise was shown to induce autophagy in; muscle, liver, pancreas, adipose tissue, and the brain (14). These findings have raised questions of whether autophagy may, in part, mediate the beneficial effects of exercise in retarding neurodegeneration and increasing physiological parameters as well as metabolic benefits. Studies of autophagy in human PBMCs are extremely rare and, consequently, makes comparing our *in vivo* findings with skeletal muscle and mouse studies problematic.

Heat shock proteins are a highly conserved family of protein chaperones that have numerous protective roles and enhance the ability of cells to survive (8). Heat shock proteins accumulate intracellularly as a result of stressors such as hyperthermia or ischemia and also participate in cellular protein homeostasis in non-stress circumstances (26). We measured heat shock protein 70 (HSP70) and HSPA1A mRNA in fasted individuals before and following endurance exercise at two different exercise intensities in a warm environmental chamber (30˚C). No statistically significant increases in HSP70 were found following exercise which could be due to adaptations acquired by our endurance trained subjects that may have previously been exposed to heat or large amounts of physiological stress due to exercise. Our data show that 60 minutes of HIE (70% of VO2max) significantly increased core temperature from resting levels in which

an increase in HSP70 was not hypothesized. HSP70 was not statistically different from pre-exercise levels during either intensity however, an increasing trend was observed. We also did not show an increase in the related HSP mRNA marker HSPA1A. An increasing trend in HSPA1A was observed however, there were large amounts of variability in our data. A decrease in HSPA1A occurred at 1h post-MIE when compared to baseline levels. This may explain the null findings of HSP70 following MIE. These data are in concert with previous findings that HSP70 does not increase in human PBMCs within 4 hours of measurement following 1h of exercise at 70-80% of VO_2max (9).

Recently, our lab has shown that the heat shock response governs autophagy by a coordinated regulatory mechanism (9). It is postulated that the heat shock protein response holds precedence over the two systems and has the ability to interrupt autophagy activation (9). To explore this coordination we performed a Pearson correlation to investigate the relationship between HSP70 and LC3-II following endurance exercise. The correlation of HSP70 and LC3-II data during MIE resulted in an unexpected positive correlation, $r = 0.576$, $p<.001$. Following HIE the same relationship was not seen. It may be possible that the heat shock protein response has a governance threshold that does not precede autophagy in low-stress conditions such as MIE. Other co-occurrences of these proteins following heat stress have been reported. In human cell culture models (A549 cells), incubations in temperatures over 40˚C have been shown to significantly increase HSP70 in the presence of significantly increased levels of LC3-II (9). Furthermore, it was shown that heat pre-conditioning in these cells resulted in an even larger increase in LC3-II protein expression when exposed to starvation media (9).

The coordination of these two systems is extremely complex and highly situational. Cell culture models demonstrate this complication. A549 cells were transfected with HSF-1 siRNA to induce overexpression of HSP70 (9). Following viral overexpression of HSP70, cells were exposed to starvation conditions in which the starvation-induced autophagy response seen in control cells was significantly inhibited (9). Dokladny et al. also investigated the role of nuclear transcription factor HSF-1, an important regulator of heat shock protein. In their experiment HSF-1 was selectively knocked down by siRNA in A549 cells. The depletion of HSF-1 expression resulted in marked increases in autophagy under control conditions as well as amplified the autophagy response following starvation (9). Dokladny et al. also studied the *in vivo* effects of HSP70 overexpression stimulated by glutamine supplementation on autophagy following 1h of endurance exercise at 70% of VO_2max at 30˚C. These data show that autophagy can be increased following exercise under hyperthermic conditions and that increased HSP70 due to glutamine supplementation prevents the increases of autophagy seen without supplementation (9).

Our data contribute to the exciting and expanding knowledge of the health benefits associated with autophagy and exercise. The optimal time course of autophagy following endurance exercise in human PBMCs is dependent on time post-exercise; however, both MIE and HIE are capable of inducing autophagy. We did not observe significant increases in HSP70 in individuals that experienced hyperthermia in our data collection time frame. There is a positive relationship between HSP70 and LC3-II that may also be intensity dependent. The importance of this observed relationship is unknown considering there were no significant changes in HSP70. Several physiological

occurrences following exercise change circulating blood constituents in which PBMCs may be targeted or affected. It is becoming widely accepted that autophagy promotes the protective effects of exercise on morbidity and mortality, and provides energy substrates during exercise through the recycling and clearance of cytosolic components.

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CHAPTER IV

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

The research manuscript entitled "The Early Time Course of Autophagy in Human Peripheral Blood Mononuclear Cells Following Endurance Exercise" provides timeline evidence of autophagy activation and factors we believe could have important regulatory actions on autophagy. We exercised individuals in a warm environmental chamber to induced higher core temperatures and mild hyperthermia. HSP70 was measured in this time course with the ambition of comparing the autophagy and HSP homeostatic systems. We found a positive correlation between LC3-II and HSP70; however, the importance of this relationship in PBMCs is not known because there were no significant increases in HSP70 in our 4h study time course. In addition, we found that moderate-intensity exercise was also able to upregulate autophagy marker LC3-II at 1h following exercise.

Conclusions

The significant findings of this study were (1) autophagy can be stimulated at several time points following high-intensity endurance exercise, (2) moderate-intensity endurance exercise in a 30˚C environment provides a stress great enough to induce autophagy, (3) HSP70 is not induced between exercise cessation and 4h post-exercise regardless of intensity, and (4) there is a moderate, positive relationship between non-significant levels of HSP70 and LC3-II following moderate-intensity exercise.

Recommendations

Although we were able to draw associations between autophagy, IL-6, and insulin-Akt signaling, we cannot confirm that the stimulation of autophagy is stimulated by these mechanisms. Important measurements in the study of autophagy at this time are mTOR and Beclin-1. We found that mTOR was very difficult to measure in human PBMCs. We did not measure Beclin-1 due to time and financial constraints; however, this measure may show that autophagy is induced independent of mTOR regulation. It was suggested that autophagy is regulated in a "biphasic" manner. It is recommended that this biphasic flux be investigated further as we did see two peaks indicative of autophagy increase following high-intensity treadmill running. The effects of cytokine IL-6 on PBMCs and autophagy regulation remain unknown. We suggest that the effects of plasma IL-6 on autophagy in PBMCs be investigated in future experiments. When measuring autophagy in PBMCs, we recommend considering the time point of measurement. Exercise intensity should also be considered when inducing autophagy. There are many other signaling pathways, such as AMPK and growth factor signaling, that affect mTOR; we did not consider them for this study. We suggest that these pathways be explored in human PBMCs following endurance exercise. It is also unknown if high-intensity interval training, resistance training, or post-prandial stimulation of insulin affects autophagy regulation in PBMCs. The study of exercise and autophagy is novel and thus, there are numerous opportunities for future research in the physiologic responses to exercise.

APPENDICIES

A. HIPAA Form

B. Informed Consent

C. Flyer

D. Health History Questionnaire

E. Human Research Review Committee Approval

F. Data Collection Sheet

G. Departmental Scientific Review Approval

H. Supplemental Figure 11

I. Supplemental Figure 12

J. Supplemental Figure 13

APPENDIX A

UNIVERSITY OF NEW MEXICO HEALTH SCIENCES CENTER HIPAA[1] AUTHORIZATION TO USE AND DISCLOSE PROTECTED HEALTH INFORMATION FOR RESEARCH PURPOSES

Title of Study: Early Time Course of Autophagy Following Endurance Exercise in Human Peripheral Blood Mononuclear Cells Study # 13-063

Principal Investigator: **Christine Mermier, Ph.D.**
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Co-Investigators: **Kathryn Lanphere, Suzanne Schneider, Karol Dokladny, Pope Moseley, Ann Gibson, Micah Zuhl, Collin Carriker, Ethan Harris, Troy Purdom, James McCormick, Jason Beam, Hung-Sheng Hsu**

Sponsor:

1. **What is the purpose of this form?** You have been asked to take part in a research study. The consent form for this study describes your participation, and that information still applies. This extra form is required by the federal Health Insurance Portability and Accountability Act (HIPAA). The purpose of this form is to get your permission (authorization) to use health information about you that is created by or used in connection with this research.

2. **What if I don't want my personal health information (PHI) to be used in this research study?** You do not have to give this permission. Your decision not to sign this form will not change your ability to get health care outside of this research study. However, if you do not sign, then you will not be allowed to participate in the study.

3. **What PHI am I allowing to be used for this research?** The information that may be used includes: Health history questionnaire data, including history of cardiovascular disease, previous injury, medications, current diet, medications or supplements, and exercise program. Height, weight, skin % body fat, and maximal oxygen consumption will be measured. In addition blood samples will be taken for measurement of proteins, cytokines, and mRNA associated with autophagy, heat shock protein, and inflammation.

4. **Where will researchers go to find my PHI?** We may ask to see your personal information in records at hospitals, clinics or doctor's offices where you may have

[1] HIPAA is the Health Insurance Portability and Accountability Act of 1996, a federal law related to privacy of health information.

received care in the past, including but not limited to facilities in the UNM health care system.

5. **Who will be allowed to use my information for this research and why?** The researchers named above and their staff will be allowed to see and use your health information for this research study. It may be used to check on your progress during the study, or analyze it along with information from other study participants. Sometimes research information is shared with collaborators or other institutions. Your records may also be reviewed by representatives of the research sponsor or funding agency, the Food and Drug Administration (FDA) to check for quality, safety or effectiveness, or the Human Research Review Committee (HRRC) for the purposes of oversight and subject safety and compliance with human research regulations.

6. **Will my information be used in any other way?** Your information used under this permission may be subject to re-disclosure outside of the research study and be no longer protected under certain circumstances such as required reporting of abuse or neglect, required reporting for law enforcement purposes, and for health oversight activities and public health purposes.

7. **What if I change my mind after I give this permission?** You can change your mind and withdraw this permission at any time by sending a written notice to the Principal Investigator at the mailing address listed at the top of this form to inform the researcher of your decision. If you withdraw this permission, the researcher may only use and share your information that has already been collected for this study. No additional health information about you will be collected by or given to the researcher for the purposes of this study.

8. **What are the privacy protections for my PHI used in this research study?** HIPAA regulations apply to personal health information in the records of health care providers and other groups that share such information. There are some differences in how these regulations apply to research, as opposed to regular health care. One difference is that you may not be able to look at your own records that relate to this research study. These records may include your medical record, which you may not be able to look at until the study is over. The HIPAA privacy protections may no longer apply once your PHI has been shared with others who may be involved in this research.

9. **How long does this permission allow my PHI to be used?** If you decide to be in this research study, your permission to access and use your health information in this study may not expire, unless you revoke or cancel it. Otherwise, we will use your information as long as it is needed for the duration of the study

I am the research participant or the personal representative authorized to act on behalf of the participant. By signing this form, I am giving permission for my personal health information to be used in research as described above. I will be given a copy of this authorization form after I have signed it.

_____ _____ _____

Name of Research Subject Signature of Subject/Legal Representative Date

Describe authority of legal representative

_____ _____ _____

Name of Person Obtaining Authorization Signature Date

APPENDIX B

Early Time Course of Autophagy Following Endurance Exercise in Human Peripheral Blood Mononuclear Cells: STUDY# 13-063

Investigators: Christine Mermier, Ph.D., Kathryn Lanphere, MS and their associates

Why you are being invited to take part in a research study

We invite you to take part in a research study because you are between the ages of 18 and 40 and are an endurance trained runner.

What you should know about a research study

Someone will explain this research study to you.
Whether or not you take part is up to you.
You can choose not to take part.
You can agree to take part now and later change your mind.
Whatever you decide it will not be held against you.
Feel free to ask all the questions you want before you decide.

Who can I talk to?

If you have questions, concerns, or complaints, or think the research has hurt you, please contact Christine Mermier (505)-277-2658, or Kathryn (Rosie) Lanphere (505)-718-8063. You may also contact the Human Research Protections Office (505)-272-1129 if you do not want to speak with an investigator.

This research has been reviewed and approved by an Institutional Review Board. You may talk to them at (505) 272-1129 or HRPO@Salud.unm.edu for any of the following:

Your questions, concerns, or complaints are not being answered by the research team.
You cannot reach the research team.
You want to talk to someone besides the research team.
You have questions about your rights as a research subject.
You want to get information or provide input about this research.

Why are we doing this research?

We are doing this research to determine the time course of an exercise-induced biological system called autophagy in human blood cells. Autophagy is the process that contributes to recycling of cellular material such as damaged proteins and organelles. Not much is known about exercise and autophagy and even less is known about the time course of auotphagy activation after endurance exercise. We will measure markers in your blood associated with autophagy, the heat shock response (another process associated with stabilizing proteins) , and inflammation. We are hoping to determine how moderate intensity endurance exercise activates autophagy when compared to high intensity endurance exercise.

How long will the research last?

We expect that you will be in this research study for three days in a six week period. You will participate in baseline testing on the first visit to the lab that will last around 2 hours. The second and third visits you will be asked to participate for approximately 6 hours each visit. Therefore, the total time commitment will be no more than14 hours over three days.

How many people will be studied?

We expect 15 people at the University of New Mexico and Albuquerque area will participate in this study.

What happens if I say yes, I want to be in this research?

If you agree to participate, the following things will happen:

Your participation will involve three visits to the University of New Mexico Exercise Physiology Lab. During the first visit, baseline measurements will be taken after an overnight fast and will include a treadmill maximal oxygen consumption test, and measurement of your body fat percentage. Women who are pregnant will be excluded from the study, and each female subject will be required to take a urine pregnancy test during the day of baseline measurements. You will be required to be in the lab for 2 hours on this visit. The treadmill maximal oxygen consumption test (VO_{2peak}) is an 8-12 minute running test to fatigue where the amount of oxygen you consume is measured throughout the test and the highest value of oxygen consumed is observed. During this test you will be required to wear a mouthpiece and nose clip. Body fat percentage will be measured using 3-site skinfold caliper test (women: triceps, hip, thigh and men: chest, abdominal, thigh).

The two additional visits will require 60 minutes of high intensity (70% of VO_{2peak}) or moderate intensity (50% of VO_{2peak}) treadmill running in a warm environmental chamber (86°F/30˚C). You may complete this protocol in two 30 minute bouts separated by 5 minutes for rest and rehydration. On these visits you should expect to be available to the lab for 6 hours from your arrival time. During each bout of exercise, heart rate and core temperature will be measured using a heart rate monitor around your chest, and a rectal thermometer placed 5 inches past the anal sphincter, respectively. Each trial will be terminated if core temperature reaches 104°F (40˚C). Blood markers (such as: plasma cytokines, autophagy markers, HSP 70) will be measured in a lab after each trial. The order of exercise intensities for the two trials will be randomly selected and will be separated by by at least 2 weeks.

Blood measurements will be made by collecting 20 ml (1.3 tbs.) of blood from a vein in your arm at six time points. A resting 20 ml (1.3tbs.) blood sample will be collected prior to the start of the exercise trial. Immediately after each trial a 20 ml (1.3 tbs.) blood sample will be collected via venous puncture. An additional 20 ml (1.3 tbs.) of blood will be collected via venous puncture at the following time points: 30 minutes, one hour, two hours, and four hours after each exercise trial. In total, 120 ml (7.8 tbs) of blood will be collected during each trial (rest, post exercise, 30 min post exercise, one hr. post exercise, two hrs. post exercise, and four hrs. post exercise). The total amount of blood collected will be 240 ml (15.6 tbs.) over the entire length of the study (2 x 120 ml for exercise trials).

What happens if I do not want to be in this research?

You may decide at any time not to take part in the research. There will be no adverse consequence to your decision.

What happens if I say yes, but I change my mind later?

Your participation in this study is completely voluntary. You have the right to choose not to participate or to withdraw your participation at any point in this study.

Is there any way being in this study could be bad for me?

Participation in this study may increase your risk of muscle soreness as you will be required to perform running sessions at 70% and 50% of your maximal effort for 1 hour. The risk of soreness increases at higher intensities. Blood drawing risks: You may have temporary pain and discomfort from the needle sticks, occasional bruising, sweating, feeling faint or lightheaded and in rare case there is risk of infection and tissue damage. The placement of the rectal thermometer may also cause embarrassment or discomfort. In addition, there is always a minor, very low risk of a cardiovascular event (about 1 in 10,000) when a person with cardiovascular disease performs exercise. Healthy and trained individuals have an even lower risk. Your participation is completely voluntary and you may withdraw at any point whether or not you experience any of these side effects.

There are risks of stress, emotional distress, inconvenience and possible loss of privacy and confidentiality associated with participating in a research study. For more information about risks and side effects, ask the investigator.

Will being in this study help me anyway?

We cannot promise any benefits to you or others from your taking part in this research. You may be interested in learning your VO_2max and body composition results.

What happens to the information we collect?

Efforts will be made to limit your personal information, including research study and medical records, to people who have a need to review this information. We cannot promise complete secrecy. Organizations that may inspect and copy your information include the IRB and other representatives of this organization. There may be times when we are required by law to share your information. However, your name will not be used in any published reports about this study.

Your information will be stored in a locked cabinet in the CTSC building located on the UNM North Campus. Blood specimens will be stored for 3 years after the end of data analysis. Your blood will be stored in a freezer located in the CTSC building that only the study team can access. In addition, your specimens and data will only be known by subject number, which will not be linked to your name or personal information.

Federal law provides additional protections of your personal information. These are described in an attached document.

Can I be removed from the research without my OK?

The person in charge of the research study or the primary investigator can remove you from the research study without your approval. Possible reasons for removal include circumstances where the investigators learn that the subject did not honestly answer the health questionnaire, fails to complete the study trials in 6 weeks, begins a diet that involves caloric restriction within the washout period of exercise trials, has consumed caffeine 12 hours prior to study, has consumed alcohol 24 hours prior to study, begins taking metabolic supplements within the time of testing, or fails to report to the scheduled lab appointment on more than one occasion. We may also remove you for any reason we feel it would not be safe for you to continue the study such as foot or leg pain issues, flu, or other sickness.

We will tell you about any new information that may affect your health, welfare, or choice to stay in the research.

What else do I need to know?

If you need medical care because of taking part in this research study, contact the

investigator and medical care will be made available. Generally, this care will be billed to you or your insurance. The University of New Mexico has no program to pay for medical care for research-related injury.

If you agree to take part in this research study, we will pay you $5 dollars for your baseline visit to the lab, $15 dollars for your first exercise trial, and $20 for the second exercise trial. A total of $40 may be disbursed for your time and inconvenience.

You will not be informed of the results of the research other than the results of your VO_2max and body fat tests.

Signature Block for Capable Adult

Your signature documents your permission to take part in this research.

DO NOT SIGN THIS FORM AFTER THIS DATE → April 1, 2014

Signature of subject	Date
Printed name of subject	
Signature of person obtaining consent	Date
Printed name of person obtaining consent	Form Date

APPENDIX C

HRPO#STUDY 13-063

Early Time Course of Autophagy Following Endurance Exercise in Human Peripheral Blood Mononuclear Cells

Trained Runners Needed For Research Study

We are researching an important biological function called autophagy following endurance exercise. We are looking for healthy individuals ages 18-40 that are trained endurance runners to participate. Volunteers will be offered a small compensation for their time and inconvenience.

If you are interested in being a subject in our study in the Health, Exercise, and Sports Science Department at The University of New Mexico please contact:

Kathryn Rosie Lanphere, M.S.

Cell: 505-718-8063, email: klanpher@unm.edu.

APPENDIX D

HEALTH HISTORY QUESTIONNAIRE (RESEARCH ONLY)

Subject #_____ Date___/___/___

*Phone #: home*_____ *cell*_____

Date of Birth ___/___/___ Age____ Gender____ Ethnicity_____ Phone (W)_____

email_____

Primary health care provider and health insurance_____

(Only for information/emergency contact)
Person to contact in case of emergency:
name_____

phone #_____

MEDICAL HISTORY
Self-reported: Height_____ Weight_____
Physical injuries:_____
_____

Limitations_____
_____

Have you ever had any of the following cardiovascular problems? Please check all that apply:

Heart attack/Myocardial Infarction____ Heart surgery ____Valve problems_____
Chest pain or pressure ___Swollen ankles____Dizziness_____Arrhythmias/Palpitations____
Heart murmur____Shortness of breath _____Congestive heart failure____
Have you ever had any of the following? Please check all that apply.
Hepatitis/HIV_____Depression_____Cancer (specify type)_____
Rheumatic fever_____High blood pressure_____ Thyroid problems_____
Kidney/liver disease_____Obesity_____ Total cholesterol >200 mg/dl_____
Diabetes (specify type)_____Asthma_____ HDL cholesterol <35 mg/dl_____
Emphysema_____Stroke____ LDL cholesterol >135 mg/dl_____

Do immediate blood relatives (biological parents & siblings **only**) have any of the conditions listed above? If yes, list the problem, and family member age at diagnosis.

_____
_____

Is your mother living? Y N Age at death_____ Cause_____

Is your father living? Y N Age at death_____ Cause_____

Have you ever been diagnosed with a gastrointestinal disease or disorder? Y N
Have you ever experienced a heat related illness? Y N
Do you currently have any condition not listed that may influence test results? Y N
Details_____
_____

Indicate level of your overall health. Excellent ____ Good ____ Fair ____ Poor_____
Are you taking any medications, vitamins or dietary supplements now? Y N
If yes, what are
they?_____
_____

Do you have allergies to any medications? If yes, what are they?
_____
_____

Are you allergic to latex? Y N
Have you been seen by a health care provider in the past year? Y N
If yes, elaborate
_____

Have you had a prior treadmill test? Y N. If yes, when?_____ What were the results?

Have you ever experienced any adverse effects during or after exercise (fainting, vomiting, shock, palpitations, hyperventilation)? Y N If yes,
elaborate._____
_____
_____

LIFESTYLE FACTORS

Do you now or have you ever used tobacco? Y N If yes: type
_____
How long?_____ Quantity____/day Years since
quitting_____
How often do you drink the following?
Caffeinated coffee, tea, or soda _____oz/day Hard liquor _____oz/wk
Wine _____oz/week
Beer _____oz/wk
Indicate your current level of emotional stress. High____ Moderate ____ Low____

PHYSICAL ACTIVITY/EXERCISE

Physical Activity
Minutes/Day (*Weekdays*) Minutes/Day (*Weekends*)

_____/_____ average _____/_____ average

Do you train in any activity (eg. Jogging, cycling, swimming, weight-lifting)? Y N
How well trained are you?

Vigorous Exercise (>30 Minute sessions)
_____Minutes/hours a week

WOMEN ONLY

Please check the response that most closely describes your menstrual status:
_____ Post-menopausal (surgical or absence of normal menstrual periods for 12 months)
_____ Eumenorrheic – Normal menstrual periods (~every 28 days)
_____ Amenorrheic – Absence of normal menstrual periods for at least 3 months
_____ Oligomenorrheic – Irregular menstrual periods with occasional missed cycles.

APPENDIX E

UNM HEALTH SCIENCES CENTER

Human Research Review Committee
Human Research Protections Office

April 29, 2013

Christine Mermier

cmermier@unm.edu

Dear Christine Mermier:

On 4/29/2013, the HRRC reviewed the following submission:

Type of Review: Initial Study
Title of Study: Early time course of autophagy following endurance exercise in human peripheral blood mononuclear cells
Investigator: Christine Mermier
Study ID: 13-063
Funding: None
Grant ID: None
IND, IDE, or HDE: None
Documents Reviewed: • 13-063 Mermier Consnt v.032213
- Protocol Clean,
- Data Collection Sheet.
- Health History Questionnaire.
- Recruitment Flyer 03-22-13.

The IRB approved the study from 4/02/2013 to 4/1/2014 inclusive. Before 4/1/2014 or within 30
days of study closure, whichever is earlier, you are to submit a continuing review with required
explanations. You can submit a continuing review by navigating to the active study and clicking
Create Modification / CR.
If continuing review approval is not granted before the expiration date of 4/1/2014, approval of
this study expires on that date.

Category: Full Committee
Determinations/Waivers: None
To request continuing review approval or closure, you are to submit a completed 'FORM: Continuing Review Progress Report (HRP-212) and required attachments 45 days prior to 4/1/2014.

The University of New Mexico • MSC08 4560 • 1 University of New Mexico • Albuquerque, NM 87131-0001 • Phone 505.277.1129 • Fax 505.277.0803 • **hsc.unm.edu/som/research/hrrc** • BMSB B71

Approval of this protocol will expire if the IRB does not grant continuing review approval before
4/1/2014.

In conducting this study, you are required to follow the requirements listed in the Investigator
Manual (HRP-103), which can be found by navigating to the IRB Library within the IRB system.

Sincerely,

Mark Holdsworth, PharmD
Executive Chair

APPENDIX F

Early time course of autophagy following endurance exercise in human peripheral blood mononuclear cells

Study Contact: Rosie Lanphere, klanpher@unm.edu, 505-718-8063

HRPO# 13-063

DATA COLLECTION SHEET

Date: _____ Time: _____ Subject#: _____

Age/Sex (yrs): _____ Ht (cm): _____ Wt (kg): _____

Skinfold Measurements (mm): Triceps or chest ____ ____ ____ = _____ (avg)
Suprailiac or abdominal ____ ____ ____ = _____ (avg)
Thigh ____ ____ ____ = _____ (avg)

Trial Type (circle one): 50%VO2peak 70%VO2peak

Calculated: 70% VO_2max : _____ 50% VO_2max : _____
Hrmax:_____

Time (minute) 60-min protocol	Heart Rate	VO2 (ml.kg.min)	Core Temp (rectal)	RPE	Comments/Speed
Resting					
0-5 warm-up					
6-10					
11-15					
16-20					
21-25					
26-30					
31-35					
36-40					
41-45					
46-50					
51-55					

56-60					

1. Subject may drink ad libitum (at will)
2. Collect 20ml of blood post exercise, 30 min, 1 hr post, 2hrs post, 4hrs post

Hematocrit data: Study # 13-063, Contact: Rosie Lanphere 505-718-8063
Subject #_____ Date: _____

Hematocrit measurements		Plasma (cm)	Hematocrit (cm)	Total Volume (cm)	
Pre-exercise	1				
	2				
	3				
Post-exercise	1				
	2				
	3				
30 min post	1				
	2				
	3				
60 min post	1				
	2				
	3				
2 h post	1				
	2				
	3				
4 h post	1				
	2				
	3				

Pregnancy test –circle one (females only): Positive Negative

APPENDIX G

Departmental Scientific Review Form

UNM | Human Research Protections Office

The HRPO expects that a scientific review be conducted at the department level by either a Chair or designee with appropriate expertise in the given study area.

Human Research Protections Office
University of New Mexico Health Sciences Center
Phone: (505) 272.1129 Fax: (505) 272.0803
hrpo@salud.unm.edu

Study & Contact Information

Study Title: Early Time Course of Autophagy Following Endurance Exercise in Human Peripheral Blood Mononuclear Cells

Principal Investigator: Christine Mermier, Ph.D./cmermier@salud.unm.edu (Name/Email)

Coordinator: _____ (Name/Email)

Department Review Checklist

1. * Verify that the following criteria are met:

- [X] The rationale for the study is clearly stated and the rationale is scientifically sound.
- [X] The specific aims and objectives of this study are clearly stated and measurable.
- [X] The standards for conducting this research are consistent with any guidelines of relevant professional associations and scholarly disciplines.
- [X] The research uses procedures that are scientifically sound and appropriate to the purpose of the study with the least amount of risk.
- [X] The study design is adequate to achieve the specific objectives of this study and the proposed participant population is appropriate.
- [X] The data to be collected are necessary to the meet the objectives of the study.
- [X] Adequate literature review has been done to support and justify this study.
- [X] Statistical considerations, including sample size and justification, estimated accrual and duration, and statistical analysis are clearly described and are adequate to meet the study objectives.
- [X] The principal investigator & any other investigator involved in this research have sufficient resources/facilities to carry out the research.
- [X] The principal investigator & any other investigator involved in this research are qualified by training and experience to personally conduct and/or supervise the research described in the protocol.
- [X] The principal investigator & any other investigator involved in this research have completed all institutional credentialing requirements, if any, to conduct the research.

Provide an explanation for each box left unchecked:

_____

2. * Reviewer Determination:

- [X] Approved
- [ ] Disapproved

3. Notes:

I certify that the statements herein are true, complete, and accurate to the best of my knowledge, and accept the obligation to assure compliance with all applicable federal regulations and state laws, institutional policies and procedures, and the requirements and determinations of the UNM Human Research Protections Office (HRPO) with respect to this research.

By signing, I hereby approve this project, based on my review:

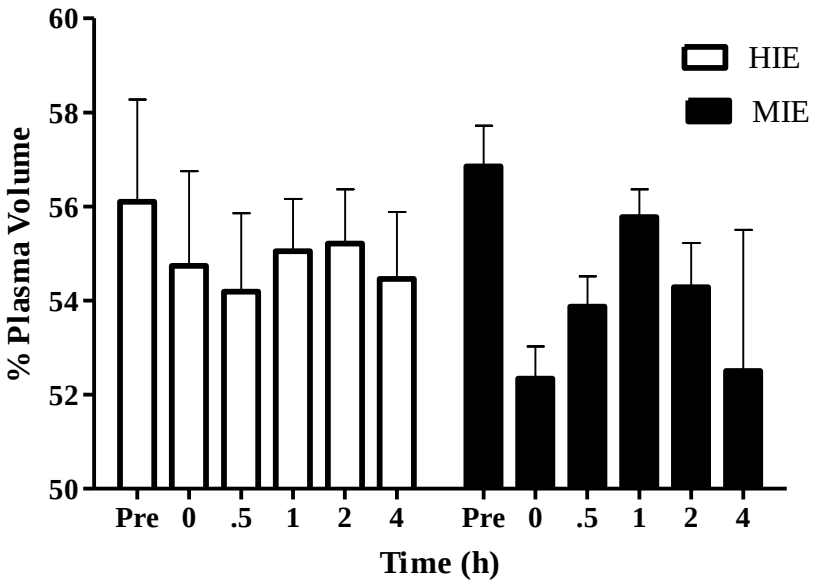
Todd Seidler, Ph.D, Department Chair — Name of Department Chair or Designee & Title

Health, Exercise, & Sports Science — Department

Todd L. Seidler — Signature of Department Chair or Designee

2/18/13 — Date

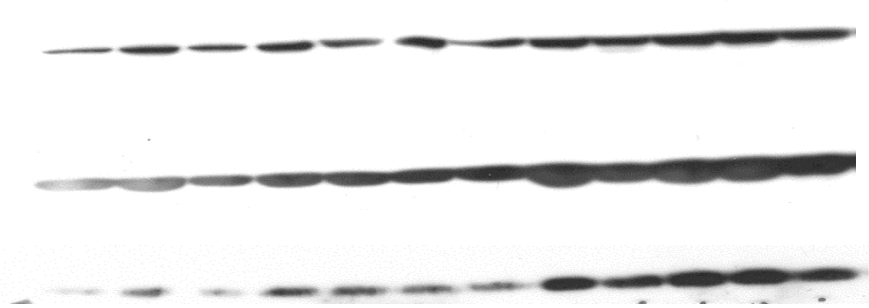
APPENDIX H



Supplemental Figure 12. Percent changes in plasma volumes. Percent of plasma volume change from baseline for all subjects during two exercise intensities. Data shown are the percent of plasma volumes during the pre-exercise and hours post-exercise No statistically significant differences were found.

APPENDIX I

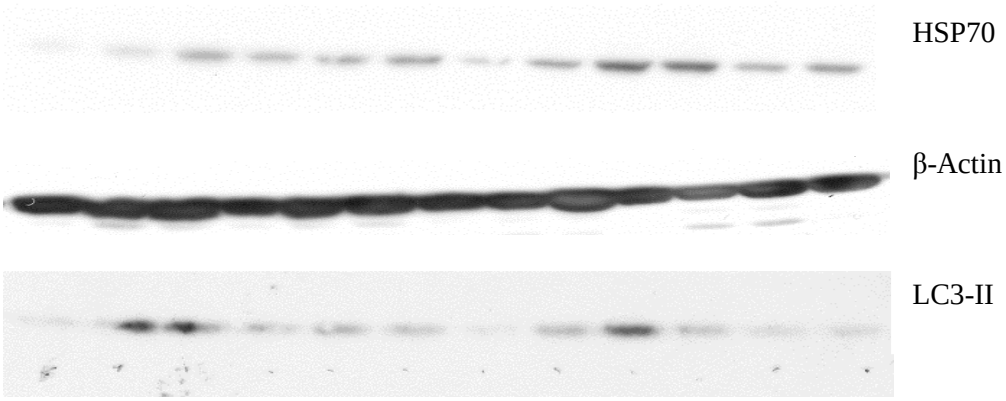
Subject A



Pre Post 30 1h 2h 4h Pre Post 30 1h 2h 4h

[----------70% VO_2max ----------] [---------50% VO_2max ---------]

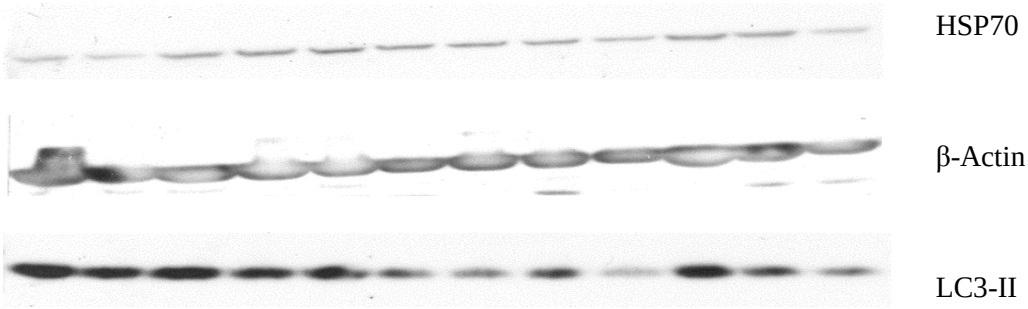
Subject B



Pre Post 30 1h 2h 4h Pre Post 30 1h 2h 4h

[----------70% VO_2max ----------] [---------50% VO_2max ---------]

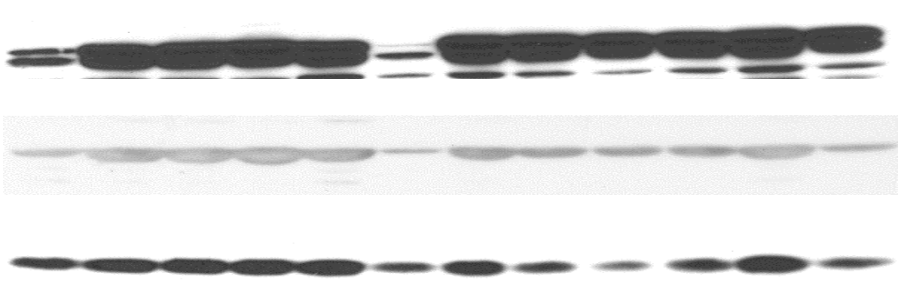
Subject C



Pre Post 30 1h 2h 4h Pre Post 30 1h 2h 4h

[----------70% VO_2max ----------] [---------50% VO_2max ---------]

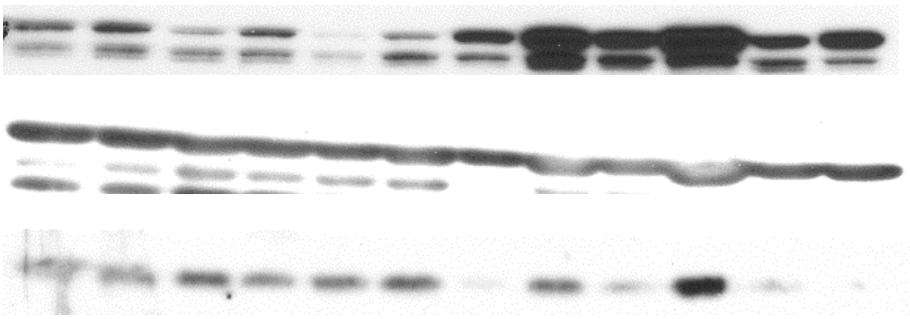
Subject D



Pre Post 30 1h 2h 4h Pre Post 30 1h 2h 4h

[----------70% VO_2max ----------] [---------50% VO_2max ---------]

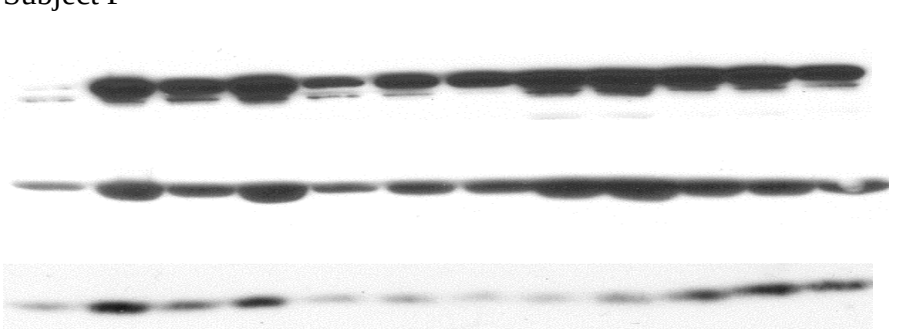
Subject E



Pre Post 30 1h 2h 4h Pre Post 30 1h 2h 4h

[----------70% VO_2max ----------] [---------50% VO_2max ---------]

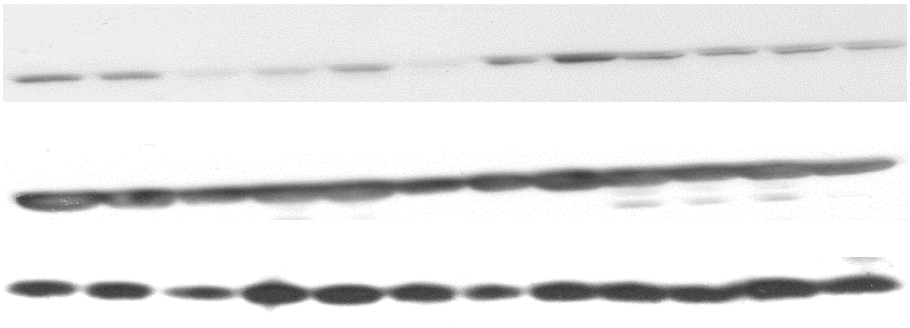
Subject F



Pre Post 30 1h 2h 4h Pre Post 30 1h 2h 4h

[----------70% VO_2max ----------] [---------50% VO_2max ---------]

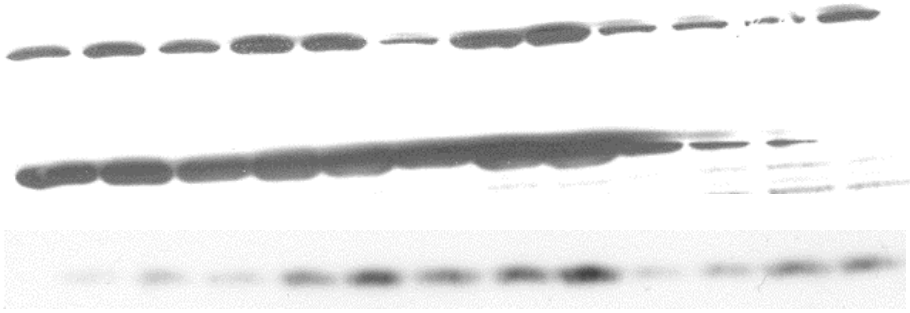
Subject G



Pre Post 30 1h 2h 4h Pre Post 30 1h 2h 4h

[----------70% VO_2max ----------] [---------50% VO_2max ---------]

Subject H

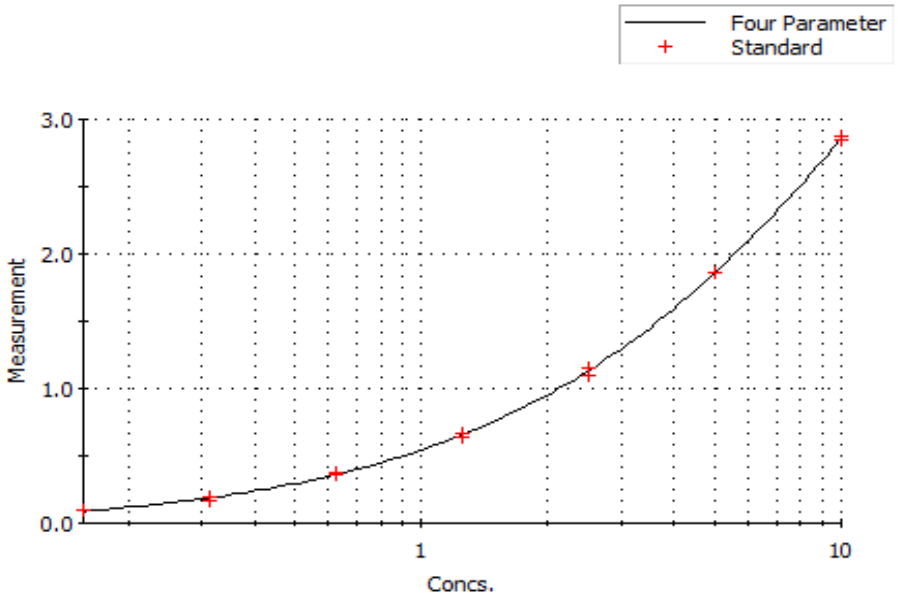


Pre Post 30 1h 2h 4h Pre Post 30 1h 2h 4h

[----------70% VO_2max ----------] [---------50% VO_2max ---------]

Western Blots for 8 subjects.

APPENDIX J



Supplemental Figure 13. Four Parameter Standard Curve for IL-6 ELISA Calculations.

MSE = 0.000207249, R^2 = 0.9998.