The effect of acute aerobic exercise and rapamycin treatment on autophagy and the heat shock response in peripheral blood mononuclear cells of prediabetics

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DISSERTATION

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ABSTRACT

**Objectives**: Recently, a malfunction of the autophagic pathway has been implicated with impaired glucose metabolism and progression from prediabetes to type 2 diabetes. Further, an interconnection has been suggested between the autophagy and heat shock protein (HSP) systems. The aims of the present study were to investigate the effects of exercise and rapamycin treatment (RAPA) on the autophagic process and HSP in peripheral blood mononuclear cells (PBMCs) from prediabetics compared to controls.

**Methods**: Two groups matched for age and sex served as subjects and consisted of six prediabetic (42.4±11.7) and six controls (44.4±11.9). Subjects exercised at 50% of VO_{2\text{max}} for 60 min with 5 min of rest dispersed every 20 min. PBMCs were isolated pre-exercise, immediately post-exercise, and 4 hr after exercise recovery. Additional PBMCs were either exposed to bafilomycin (BAF), rapamycin with bafilomycin (RAPA), or no treatment (NT) with vehicle (dimethylsulfoxide). Proteins and mRNA were analyzed via western blot and quantitative real-time polymerase chain reaction. **Results**: Exercise increased autophagy immediately post-exercise and recovered 4 hr after exercise in control subjects but not in prediabetics. Prediabetic and control subjects both increased autophagy following RAPA treatment; however, a significantly impaired response was observed in prediabetics when compared to controls. No significant differences in HSP72 response was observed. **Conclusions**: Our results indicate an impairment in autophagic flux but not HSP in PBMCs from prediabetics when compared to controls in response to both exercise and RAPA treatment. Future methods of autophagic upregulation should be investigated to spare malfunctions in autophagy in prediabetics.
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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
µg/ml: microgram per milliliter
µl: microliter
µmol: micromole
ADA: American Diabetes Association
ADP: adenosine diphosphate
AKT: protein kinase B
AMPK: AMP-activated protein kinase
ANOVA: analysis of variance
ATG: autophagy related
ATP: adenosine triphosphate
BAF: bafilomycin
BF%: body fat percentage
cDNA: complementary deoxyribonucleic acid
cm: centimeters
DMSO: dimethylsulfoxide
ER: endoplasmic reticulum
FAK: focal adhesion kinase
FIP200: focal adhesion kinase interacting protein
FoxO3: forkhead box protein O3
GLUT4: glucose transporter 4
H2O: water
HbA1c: hemoglobin A1c
hIAPP: human islet amyloid polypeptide
Hr: hours
HR: heart rate
HRP: horseradish peroxidase
HRPO: human research review committee
HSP: heat shock protein
HSPA1A: heat shock protein family A member 1A
HSP72: heat shock protein 72
Kcal: kilocalorie
kg: kilogram
LAMP2: lysosome-associated membrane protein 2
LC3: microtubule associated light chain 3
LC3-I: microtubule associated light chain 3-1
LC3-II: microtubule associated light chain 3-2
LPS: lipopolysaccharide
M: molar
MAP1LC3B: microtubule associated protein 1 light chain 3 beta
Mg: milligram
Min: minutes
mM: millimolar
mTOR: mammalian target of rapamycin
n: number of subjects
NaCl: sodium chloride
Nrf2: nuclear factor erythroid
NT: no treatment
P62/SQSTM1: p62/sequestosome-1
PBMC: peripheral blood mononuclear cell
PBS: phosphate buffered saline
qRT-PCR: quantitative real time polymerase chain reaction
RAPA: rapamycin
ROS: Reactive oxygen species
SD: standard deviation
SEM: Standard error of the mean
Tris: tris (hydroxymethyl) aminomethane
ULK1: UNC51-like kinase
UPR: unfolded protein response
VO2 max: maximal oxygen consumption
Yr: year
CHAPTER 1

Introduction

The prevalence of diabetes mellitus is increasing globally every year and has become a universal health problem that affects all socioeconomic classes and populations. According to the International Diabetes Foundation Atlas, the estimated diabetes prevalence in 2017 was 425 million, with over 641 million people predicted to be living with diabetes by the year 2040 [1]. Type 2 diabetes comprises a group of metabolic disorders which result in cardiovascular associated complications [2], such as coronary artery disease, as well as microvascular disorders including nephropathy, retinopathy and neuropathy [3]. Additionally, type 2 diabetes can lead to impaired reproductive performance [4] and contributes to increased frequency of fetal complications [5]. Progression to type 2 diabetes occurs with chronic insulin-resistance which leads to a rise in pancreatic β-cell activity in an effort to compensate for low insulin sensitivity and an increase in glucose tolerance which can lead to β-cell dysfunction [6]. Prediabetes represents a period of higher blood glucose concentrations than normal but lower than the threshold for type 2 diabetes, making a high-risk state for type 2 diabetes development [7]. The American Diabetes Association (ADA) considers individuals at risk for developing type 2 diabetes (leaving them in a prediabetic state) by having a hemoglobin A1c (HbA1c) value between 5.7-6.4%, with type 2 diabetes being diagnosed at an HbA1c greater than 6.4% [8]. According to the ADA, up to 70% of individuals with prediabetes will eventually develop type 2 diabetes in their lifetime [8].

Macroautophagy (herein referred to as autophagy) is an important lysosome degradation pathway through which damaged organelles and macromolecules are degraded within the cell [9, 10]. Autophagy is a process present in all eukaryotes which involves the removal of protein
aggregates and damaged or excess organelles in an effort to maintain intracellular homeostasis [10]. For instance, autophagic disruption results in the accumulation of abnormal mitochondria in adult tissues resulting from an accumulation in reactive oxygen species (ROS) [11, 12].

Autophagy plays a physiological role in mammalian biology, the disruption or dysfunction of which leads to the pathophysiology of a variety of diseases. Dysfunctional autophagy leads to the accumulation of damaged molecules and organelles which may contribute to neurodegenerative disorders [13, 14], cardiomyopathy [15], and cancer [16].

The beneficial effects of exercise in prediabetes and type 2 diabetes have been well established [17]. Exercise is known to increase autophagy in healthy individuals [18]; however, little is known about the effects of exercise on autophagy in prediabetics or those with type 2 diabetes. To date, only one study has examined the impact of aerobic exercise (60 min cycling at 50% VO$_{2\text{max}}$) on type 2 diabetics which showed no impairment of autophagic flux during or 3h post-exercise in skeletal muscle [19]. A comparison of basal autophagy showed an adaptation in which autophagy is able to engage under chronic hyperglycemia in human skeletal muscle of obese individuals and type 2 diabetics; however, the exercise responses were not examined [20]. Thus, further comparisons of the autophagic response in the presence of insulin-resistance or diabetes are limited to animal models. In high-fat diet fed mice, a single bout of exercise (1-hour treadmill running) increased the activity of autophagy in skeletal muscle and increased insulin-sensitivity, while 6-weeks of treadmill running increased basal levels of autophagy in both high-fat diet-fed mice and control mice [21]. Moderate physical activity has been shown to increase basal hepatic autophagy in diet-induced obese mice, which may act as protection from hepatic fat accumulation [22]. However, 8-weeks of treadmill exercise had no effect on soleus muscle autophagy, despite improvements in body weight, body fat, and insulin-resistance in high-fat diet
induced obese rats [23]. High intensity aerobic exercise training (8 days, 2*24 min, 50 to 90% max aerobic speed) on a treadmill showed no difference in basal autophagy modulation despite an increase in insulin sensitivity in mice [24]. After an extensive review of the literature, no known studies have been found that examine the impact of aerobic exercise on markers of autophagy in prediabetic human peripheral blood mononuclear cells (PBMC). More research is necessary to establish a connection between autophagy and exercise in prediabetics, especially considering that these individuals are at high risk for developing type 2 diabetes.

Heat shock proteins (HSP) represent a family of proteins named based on their molecular weight and decreased expression (specifically, HSP72) and may play a role in the development of type 2 diabetes and insulin-resistance [25]. While not fully understood, it is thought that HSP72 expression may interact directly with glucose transporter 4 (GLUT4) translocation and thereby affect insulin sensitivity in skeletal muscle [25]. However, conflicting evidence has also shown no reduction in GLUT4 expression in patients with type 2 diabetes vs controls [26]. Additionally, HSP72 may be involved in protecting against insulin-resistance induced by obesity through increased oxidative metabolism in skeletal muscle [27] and by blocking inflammatory signaling proteins such as inhibitor of kappaB kinase, tumor necrosis factor alpha, and c-jun amino terminal kinase [28]. Further, there is evidence that HSPs may interact with autophagy [18, 29]; however, this has not been established in those with prediabetes.

**Problem Statement**

With increasing worldwide prevalence of prediabetes and type 2 diabetes, there is need for novel therapeutic targets to assist in disease outcomes [30]. Increasing evidence suggests that autophagy may play an important role in the prevention of diabetic related conditions such as diabetic neuropathy, diabetic nephropathy, and metabolic syndrome [31, 32]. As there is a
substantially high risk for those with prediabetes to develop type 2 diabetes (approximately 70%) [8], it is important to investigate mechanisms that may aid in the prevention of progression to type 2 diabetes. No studies were found examining the impact of acute aerobic exercise on markers of autophagy in PBMCs of prediabetic individuals. Further, the ability of prediabetics to upregulate autophagy in the presence of nutrient deprivation (simulated with known autophagy inducer rapamycin) has not been established in prediabetics. Lastly, the interconnection between autophagy and the heat shock protein system has not been previously examined in prediabetic individuals.

**Study Objectives**

The purposes of the current study were: 1) to determine if there are differences in autophagy activation between prediabetic individuals and controls in response to acute aerobic exercise; 2) to determine differences in autophagy activation between prediabetic individuals and controls in response to rapamycin treatment; 3) to examine the interconnection between autophagy and heat shock protein 70 (HSP 70) in response to acute aerobic exercise in prediabetic individuals compared to controls; and 4) to examine the interconnection between autophagy and heat shock protein 70 in response to mammalian target of rapamycin (mTOR) inhibition (rapamycin treatment) in prediabetic individuals compared to controls. To accomplish these objectives, autophagy protein markers LC3-II/β-actin, LC3-II/I, and p62/β-actin ratios and their respective genes (MAP1LC3B and SQSTM1/p62, normalized to β-actin) were examined to represent autophagic flux. To examine the interconnection of autophagy with the heat shock protein system, HSP72 protein and the HSPA1A gene was examined and normalized to β-actin.

**Hypotheses**

We hypothesize that:
1) Baseline autophagy levels PBMCs in those with prediabetes will be lower than in control subjects. Rationale: This is based on animal models that have shown lower basal autophagy in insulin-resistant mice [21].

2) Acute aerobic exercise will upregulate autophagy in PBMCs in prediabetic individuals when compared to resting conditions. Rationale: While regulation of autophagy has not been examined in prediabetic human PBMCs after a bout of acute exercise, it has been shown that autophagy is upregulated in mice skeletal muscle after treadmill running [21].

3) Acute aerobic exercise will upregulate autophagy significantly more in control subjects than in prediabetic subjects. Rationale: As defective autophagy may contribute to insulin-resistance, it is thought that the acute autophagic response will be blunted in prediabetic individuals [33].

4) Rapamycin treatment will significantly upregulate autophagy in PBMCs of both prediabetic individuals and controls. Rationale: Though this has yet to be studied in PBMCs, upregulation of autophagy with rapamycin has been confirmed in insulin-resistant pancreatic β-cells [34].

5) Rapamycin treatment will upregulate autophagy greater in control PBMCs than in those of prediabetic individuals. Rationale: it is thought that the autophagic response in prediabetic PBMCs will be lower than control PBMCs due to deficient autophagy present in insulin-resistant cells [21].

6) Basal HSP levels will be lower in prediabetic individuals when compared to controls. Rationale: the heat shock response has been shown to be lower in states of insulin-resistance, thus we believe basal HSP levels will be lower in prediabetic PBMCs [35].
7) The HSP response will be blunted in prediabetic individuals when compared to controls in response to acute aerobic exercise. Rationale: as the heat shock response is blunted in insulin-resistant cells, it is expected that HSP levels will be lower in prediabetic cells in response to exercise [35].

Scope of the Study

Twelve adult participants (6 females and 6 males) between the ages of 27-61 years, who were free of known illness completed an initial maximal oxygen consumption (VO\textsubscript{2max}) test to determine cardiorespiratory fitness level and the workload for the subsequent aerobic exercise trial. Participants were age and sex matched and tested for prediabetes as determined from an initial hemoglobin A1C (HbA1C) test and were categorized as either prediabetic (HbA1C between 5.7-6.4%) or control (HbA1C <5.7%) based on the HbA1C results. Following a minimum 48-hour rest period after the VO\textsubscript{2max} test, participants underwent an aerobic exercise session which consisted of cycling for 60 minutes (3 bouts of 20 minutes, with 5-minute rest periods between bouts) at 50% of VO\textsubscript{2max}. Blood was drawn pre-, post-, and 4-hours post-exercise to determine difference in autophagy and heat shock protein activation in peripheral blood mononuclear cells (PBMCs) between prediabetic individuals and controls. Additional PBMCs were isolated and treated with either vehicle (dimethyl sulfoxide-DMSO), bafilomycin in DMSO (BAF), or rapamycin in DMSO + BAF (RAPA) to determine the effect of a known autophagy activator (RAPA) on PBMCs of prediabetic individuals when compared to controls.

Assumptions

The following assumptions were identified in this study:

1. Prior to each visit, the participant did not perform any vigorous exercise for 24 hours and did not ingest caffeine or alcohol for 12 hours.
2. The subjects ate the provided nutrient bar 2-hours prior to the aerobic exercise trial.
3. All subjects were free of known illness prior to all trials.
4. All subjects completed the maximal exercise test to volitional exhaustion.

Limitations
1. While markers of autophagy including LC3-I, LC3-II, and p62/SQSTM1 are commonly used as indications of autphagic flux [36], they are the only proteins and genes used in this study to indicate changes in autophagy. Further, HSP72 is used as the only marker of the heat shock response.
2. Markers of autophagy and the heat shock response are not measured beyond 4-hours post aerobic exercise.
3. All autophagy and heat shock measurements are derived from PBMCs which may not be representative of other insulin sensitive tissues.
4. All participants ate the same standardized meal prior to exercise; however, this was not adjusted for body weight.

Significance of the Study

This study compared the differences in autophagy and the heat shock response in human PBMCs between individuals with prediabetes and controls in response to an acute bout of aerobic exercise. It is important to determine if autophagic impairment exists in response to exercise in prediabetics as recent reports have indicated defective metabolic adaptations to exercise in type 2 diabetes [37, 38], suggesting a resistance to the beneficial effects of exercise [39]. To further understand the underlying differences in autophagy between prediabetic and control individuals, PBMCs were treated with a known autophagy upregulator (rapamycin)
through mTORC1 inhibition [36]. A secondary objective of this study was to determine the interconnection between autophagy and the heat shock response following acute aerobic exercise and rapamycin treatment. To our knowledge, this is the first study to examine these responses in human PBMCs.

**Definitions**

*Macroautophagy (autophagy):* Bulk process of degradation and cycling which involves the removal of protein aggregates and damaged or excess organelles in an effort to maintain intracellular homeostasis.

*mTOR:* mammalian target of rapamycin; a serine/threonine protein kinase that regulates cell growth and proliferation.

*Heat shock response:* Response of the heat shock protein system which involves a series of proteins named by their molecular weight. This response involves a cellular repair mechanism which is initiated in response to disruptions in cellular homeostasis.

*Insulin-resistance:* pathological condition in which cells fail to respond normally to insulin.

*Microtubule-associated protein I/II light chain (LC3):* Marker used to measure autophagy activation.

*Peripheral blood mononuclear cells:* also termed white blood cells or blood leukocytes. These are immune cells found within the human body.

*Prediabetes:* precursor stage for those at high risk of progression to type 2 diabetes. Characterized by having an HbA1c between 5.7% and 6.4%.

*Type 2 diabetes:* long-term metabolic disorder that is characterized by high blood glucose, insulin resistance, and relative lack of insulin.
**P62/sequesterome1**: A marker used to determine autophagy activation. A cargo protein that targets other proteins to bind for selective autophagy.

**Heat shock protein 72**: Inducible isoform of HSP70 that aids in cell survival in stressful conditions. It is also a protein markers used to identify the heat shock response.

**HSPA1A**: mRNA gene that encodes for HSP72 protein synthesis.

**MAP1LC3B**: gene that encodes for LC3 protein synthesis.

Inhibitor of kappaB kinase: enzyme complex that is involved in propagating the cellular response to inflammation.

Tumor necrosis factor alpha: signaling cytokine involved in systemic inflammation and is involved in the acute phase reaction of inflammation.

C-jun amino terminal kinase: Plays a role in T cell differentiation and cellular apoptosis pathway and is thought to contribute to the inflammatory response.
References


CHAPTER 2

This chapter presents a review article, entitled “insert title” which will be submitted for publication in the “Canadian Journal of Diabetes”. It is authored by James J. McCormick, Karol Dokladny, Fabiano Amorim, Len R. Kravitz, and Christine M. Mermier. The manuscript follows the formatting guidelines of the journal.
Title: Implications of autophagy in type 2 diabetes

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Abstract

Autophagy is a crucial cell survival mechanism which acts to degrade and recycle intracellular proteins, as well as, old or damaged organelles to maintain normal cellular function. Dysfunctional autophagy leads to the accumulation of damaged molecules and organelles which may contribute to the pathophysiology of neurodegenerative disorders, cardiomyopathy, and various cancers. Recent evidence suggests a malfunction of autophagy may have implications in the development of insulin-resistance and type 2 diabetes disease progression through impairments in β-cell function. In this review, we explore the role of autophagy in type 2 diabetes disease progression. We also examine the function of autophagy in pancreatic β-cells preservation. Further, the protective effects of enhanced autophagic functioning are examined in response to chronic inflammation. Lastly, we examine methods of autophagy upregulation, including pharmacological therapies and exercise-mediated autophagy responses.
Introduction

The prevalence of diabetes mellitus is increasing globally every year, which has become a pervasive health problem that affects all socioeconomic classes and populations. According to the International Diabetes Foundation Atlas, the estimated diabetes prevalence in 2017 was 425 million, with over 641 million people predicted to be living with diabetes by the year 2040 (1). Diabetes mellitus is a condition involving a chronic elevation of blood glucose levels (hyperglycemia) and is categorized into two types: type 1 and type 2. Type 1 diabetes is associated with the destruction of pancreatic beta cells resulting in insufficient insulin production, while type 2 diabetes includes a range of metabolic disorders, such as insulin resistance and obesity, which ultimately lead to hyperglycemia (90). While both types of diabetes increase the risk for the progression of microvascular disorders including nephropathy, retinopathy and neuropathy, the remainder of this review will be focused on type 2 diabetes (29, 78). Type 2 diabetes comprises a group of metabolic disorders which result in cardiovascular associated complications (4) such as coronary artery disease, hypertension, and stroke as well as impaired reproductive performance (16). Therefore, there is a need for the development of novel therapeutic targets to treat the underlying mechanisms resulting in diabetes disease progression. One such target may be that of autophagy, which has become a mechanism of interest in recent years.

Autophagy is an important lysosome degradation pathway through which damaged organelles and macromolecules are degraded within the cell (49, 50). Autophagy is a process present in all eukaryotes and can be divided into three main types which include chaperone-mediated autophagy, microautophagy and macroautophagy. Chaperone-mediated autophagy involves the selective degradation of specific cargo proteins that are recognized and delivered to
the lysosome by a chaperone complex, while microautophagy involves the nonselective sequestration of cytoplasmic components directly into the lysosome (47). This review will primarily focus on the process of macroautophagy (herein referred to as autophagy) which is a cellular process that is crucial in the removal of protein aggregates and damaged or excess organelles in an effort to maintain intracellular homeostasis (50). Thus, the process of autophagy may act as a protector of cells against various stressors and function as a quality control mechanism in response to routine cellular stressors. However, autophagy can also lead to non-apoptotic cell death or type 2 programmed cell death (12, 39, 68). Therefore, autophagy can act as an adaptive response depending on the cellular and environmental context, which can serve as a cellular protective mechanism or promote cell death.

Autophagy plays a physiological role in mammalian biology, the disruption or dysfunction of which leads to the pathophysiology of a variety of diseases. For instance, autophagic disruption results in the accumulation of abnormal mitochondria in adult tissues (44, 69). Dysfunctional autophagy leads to the accumulation of damaged molecules and organelles which may contribute to the pathophysiology neurodegenerative disorders (27, 43), cardiomyopathy (63), and cancer (14). Further, there is accumulating evidence that supports a direct role for autophagy in the aging process (3, 10, 26, 47, 81).

The Role of Autophagy in the Pathophysiology of Type 2 Diabetes

Autophagy induction is primarily mediated under two conditions: 1) under starvation conditions autophagy supplies nutrients in the form of amino acids for ATP synthesis through the removal of misfolded proteins and damaged organelles and, 2) under conditions of cellular stress (20). Autophagy mediation primarily occurs through UNC51-like kinase (ULK1). During periods of nutrient excess, mammalian target of rapamycin (mTOR) complex 1 (mTORC1) is
activated and incorporated into a complex with ULK1, autophagy related protein 13 (Atg13) and FAK-family (focal adhesion kinase-family) interacting protein (FIP200), which results in phosphorylation of ULK1, rendering it inactive and subsequently inhibiting autophagy (61). During starvation conditions, mTORC1 is dephosphorylated and dissociates from the ULK complex, thereby dephosphorylating ULK1 and becoming enzymatically activated. This leads to phosphorylation of Atg13 and FIP200 which causes induction of autophagy (7). During periods of nutrient surplus, there is an influx of insulin which also acts to inhibit autophagy through two primary mechanisms: 1) by activating mTORC1 resulting in inhibition of ULK1 (56) and, 2) by activating protein kinase B, thereby inhibiting Forkhead box protein O3 (FoxO3), which is a transcription factor that promotes autophagy-related (Atg) gene expression (18).

Insulin-resistance is often regarded as the primary cellular defect which contributes to the progression and development of type 2 diabetes (37, 71). Insulin-resistance is a condition where insulin-induced glucose uptake is impaired in insulin-sensitive tissue, such as skeletal muscle, fat, and the heart (89). The development of insulin-resistance has several proposed mechanisms including chronic inflammation (86), mitochondrial dysfunction (62), hyperinsulinemia (88), and hyperlipidemia (8), many of which are associated with obesity and aging (89). Long-term insulin resistance can occur prior to the development of type 2 diabetes and leads to an increase in the activity of pancreatic β-cells to compensate for the reduction in insulin sensitivity and impaired glucose tolerance. This leads to a subsequent decline in insulin production by pancreatic β-cells and β-cell dysfunction which consequently results in type 2 diabetes (11, 32). Cell organelles which include mitochondria and the endoplasmic reticulum play a crucial role in survival as well as with insulin action/sensitivity, and rely heavily on autophagy to maintain normal cellular function (9, 48, 54, 65).
Persistently high glucose concentrations, as seen in type 2 diabetes, lead to imbalances in antioxidant capacity resulting in mitochondrial oxidative stress and injury (24). Impairment of autophagy results in the accumulation of dysfunctional mitochondria within the cell (79). Consequently, because mitochondria are the primary site of reactive oxygen species production (ROS), dysfunctional autophagy may lead to the accumulation of ROS, which has been implicated as a contributor to insulin resistance (33, 66).

**Autophagy in Pancreatic β-Cell Maintenance**

There are several mechanisms in which autophagy aids in the maintenance of normal pancreatic β-cell function. Crinophagy is a specialized form of autophagy which is crucial in the maintenance of intracellular insulin levels (75). During periods of low intracellular glucose levels, crinophagy is increased and intracellular insulin levels decrease, while high glucose levels result in inhibition of insulin degradation (46, 76). Therefore, crinophagy is a chronically activated mechanism through which insulin stores are kept at optimal levels in an effort to maintain β-cell insulin secretory capacity (83).

One of the key homeostatic roles of autophagy is the removal of damaged or dysfunctional cell organelles, such as mitochondria. Insulin secretion is dependent on increases in intracellular ATP/ADP ratio, making autophagy-dependent maintenance of mitochondria crucial to cellular insulin homeostasis (55). Mitophagy is another form of autophagy that acts to degrade dysfunctional or damaged mitochondria in the pancreas and elsewhere, thereby one function is to prevent the accumulation of depolarized mitochondria and to aid in the maintenance of normal β-cell function (82).

Under periods of both a normoglycemic and hyperglycemic state, there is heavy demand for protein folding on the endoplasmic reticulum (ER) in the pancreatic β-cells, due to the
necessity to continually synthesize insulin to attempt to maintain a euglycemic state. During periods of heavy protein synthesis, there can be the accumulation of misfolded proteins which cause ER stress and the aggregation of proteins within the cytosol leading to a disruption of cellular function (7). Accumulation of misfolded proteins causes the initiation of an unfolded protein response (UPR) which causes a decrease in the translation of proteins (52). Autophagy assists in this response through the degradation of misfolded or unfolded proteins which allows the β-cell to maintain normal cell function (38).

**Autophagy in β-cell Dysfunction**

As previously discussed, progression to type 2 diabetes is characterized by insulin-resistant cells and over-secretion of insulin via pancreatic β-cells (89). This places undue stress on the β-cells which leads to ER stress and dysfunctional protein synthesis. In normal functioning pancreatic β-cells, autophagy degrades the misfolded or unfolded proteins, assisting in the prevention of protein aggregates and subsequent ER stress (38).

Increasing evidence suggests a role of impaired autophagy in β-cell dysfunction, thereby contributing to impaired insulin secretion. While the role of autophagy in type 2 diabetes in humans is still unclear, the expression of autophagy genes in β-cells (57) and islet cells (13) has been demonstrated. Human pancreatic cells from type 2 diabetics, that were analyzed with electron microscopy, showed autophagic functioning may be altered resulting in autophagy-associated cell death of pancreatic β-cells (59). While expression of genes of molecules associated with autophagic machinery involved in the early steps of autophagy (beclin 1 and Atg1) were unaltered, there was a reduction in the transcription of lysosome-associated membrane protein 2 (LAMP2), cathepsin B and cathepsin D, which are involved in later steps of autophagy. Further, it was found that AMP-activated protein kinase (AMPK) upregulation
through the use of the drug metformin was able to correct alterations in autophagy (19), suggesting autophagy may be a potential therapeutic target for pancreatic β-cell survival.

Alterations in autophagy in β-cells have shown that defective or impaired autophagy is associated with β-cell dysfunction or death in several animal models. Knockout mice that lack Atg7 in β-cells have been shown to demonstrate increased apoptosis (36). Atg7 deficient β-cells result in a defect in insulin secretion due to autophagy-associated defective mitochondrial function resulting from lower ATP production (23). Obese Atg7 knockout mice with hyperglycemia develop severe type 2 diabetes; however, diabetes does not develop in mice with normal functioning autophagy and UPR-related genes (70).

Akita mice, which carry a proinsulin mutation, lead to severe misfolding and subsequent ER stress (5). When stimulated with rapamycin (which acts to stimulate autophagy through mTORC1 inhibition) there was a resistance to ER stress-mediated cell death, while autophagy inhibition increased cell death. Palmitate treatment causes activation of autophagy in rat and human pancreatic cells, which leads to cell death following prolonged treatment (58). However, treatment with rapamycin provided significant protection to palmitate-induced autophagy, indicating that normal functioning autophagy may be required to counter the cytotoxic effects of fatty acids in β-cells (7).

Human islet amyloid polypeptide (hIAPP) is a β-cell specific amino acid peptide that is packaged in insulin granules and secreted with insulin, is thought to be involved in carbohydrate and calcium homeostasis (85). Accumulation of hIAPP produces toxic oligomers which contribute to the development of type 2 diabetes (35). Autophagy appears to play an important role in protecting β-cells from cytotoxic effects of hIAPP oligermination. This has been shown through treatment of β-cells with hIAPP, which results in autophagy initiation, while inhibition
of autophagy in the presence of hIAPP appears to enhance the cytotoxic effects (77). Autophagy induction via pharmacological treatment of trehalose (a glucose transport inhibitor) resulted in a reduction in the accumulation of cytotoxic hIAPP oligomers and a partial reversal of the diabetic phenotype (40).

**Autophagy and Inflammation in Type 2 Diabetes**

Inflammation appears to play a key role in the development of insulin-resistance which has the potential to progress to type 2 diabetes in obese individuals (22). Obesity is associated with increased levels of endotoxins, such as lipopolysaccharides (LPS) which are increased due to enhanced intestinal permeability (67). Autophagy is stimulated by endotoxins through activation of toll-like receptors which may restrict the inflammatory response through reduced expression of proinflammatory cytokines (73, 84).

Podocytes are structures of glomerulus filtration and are located around the glomerular basement membrane in the kidney. Podocyte injury is a common pathology of glomerular disease such as diabetic nephropathy (25). Suppression of autophagy via LPS treatment induces podocyte injury, while rapamycin-induced autophagy resulted in an attenuation of the toxic effects of LPS (80). Deficiency of Atg7 genes in mice triggers an increase in lipid-associated inflammation which causes insulin-resistance (52). Taken together, these findings suggest autophagy may play a protective role in obesity-induced inflammation and insulin-resistance.

**Autophagy as a Therapeutic Target for Type 2 Diabetes**

With increasing evidence of dysfunctional autophagy contributing to diabetic associated complications, such as loss of pancreatic β-cells (59) and diabetic nephropathy (41), autophagy modulation may provide a novel therapeutic approach to improving type 2 diabetes disease outcomes. However, this may be limited to diabetic patients with altered autophagy, for which
there is currently no known method of screening (7). Several pharmacological applications are known to upregulate autophagy; however, their use as a therapy for diabetic patients may be questionable. For instance, rapamycin is a known inhibitor of mTORC1 and a potent stimulator of autophagy (42). However, rapamycin may not be effective as a therapy in diabetics due to induction of pancreatic β-cell toxicity leading to reduced β-cell function and survival (6). Further, mTORC1 inhibition via rapamycin may impact other cellular functions besides autophagy and therefore, it is premature to recommend as a therapeutic compound in type 2 diabetic patients.

Liraglutide and exendin-4 are pharmacological agents that have been shown to upregulate autophagy in autophagy-deficient β-cells by targeting glucagon-like peptide 1 signaling which has been shown to protect β-cells against fatty acid-induced cell death in mice (2, 15). Further, commonly used hypoglycemic agents including metformin and rosiglitazone have been shown to protect β-cells against fatty-acid induced apoptosis through autophagy upregulation in type 2 diabetic pancreatic β-cells (59, 87). Another pharmacological agent shown to upregulate autophagy in primates and rodents is a nuclear factor erythroid 2 (Nrf2) activator called dihydro-CDDO-trifluorethylamide which may show promise in type 2 diabetics; however, it has not been examined in humans (51).

**Exercise and Autophagy Upregulation in Type 2 Diabetes**

While there is increased interest in the impact of exercise on autophagic functioning, research examining this effect in humans in healthy individuals or type 2 diabetics is currently in its infancy. To date, only one known study has examined the effect of exercise on autophagy in type 2 diabetics (45). It was found that 60 min of cycling exercise at 70% VO2max was able to induce autophagy in the skeletal muscle of type 2 diabetic middle-aged men. The ability of
autophagy to respond to exercise in type 2 diabetics is important, as autophagy has emerged as a critical component in the adaptation of mammalian skeletal muscle (74). A disruption in exercise-induced autophagy has been shown to alter glucose metabolism during acute exercise in mice (28) and recent reports indicate that exercise-induced autophagy plays an important role in metabolic adaptations to exercise including mitochondrial biogenesis and mitochondrial content and quality (30, 31).

In healthy adults, 1 hour of moderate-high intensity aerobic exercise (70-80% of VO₂max) in a warm (30°C) environment results in a significant increase in autophagy in peripheral blood mononuclear cells (21). Ultra-endurance running (149km for greater than 18 hours) has been shown to elicit significant increases in autophagy which were also associated with a reduction in autophagy inhibitory pathways including plasma insulin, AKT and mTORC1, with subsequent upregulation of autophagic activation via the AMPK pathway (34). Conversely, a single bout of resistance exercise appears to have little to no effect on autophagy induction in skeletal muscle. Similarly, 20 min of short high-intensity aerobic exercise (81% of VO₂max) elicits no increase in autophagy in human skeletal muscle (60).

In high-fat diet fed mice, a single bout of exercise increased activity of autophagy in skeletal muscle and increased insulin-sensitivity. Further, 6-weeks of treadmill running increased basal levels of autophagy in both high-fat diet-fed mice and control mice (53). Moderate physical activity showed an increase in basal hepatic autophagy in diet-induced obese mice, which may act to protect from hepatic fat accumulation (72). Conversely, 8-weeks of treadmill exercise had no effect on soleus muscle autophagy, despite improvements in body weight, body fat, and insulin resistance in high-fat diet induced obese rats (17). High intensity aerobic exercise training (8 days, 2*24 min, 50 to 90% max aerobic speed) on a treadmill showed no difference in basal
autophagy modulation despite an increase in insulin sensitivity in mouse muscle (64). Combined, much more evidence is necessary to determine the impact of exercise on autophagy in type 2 diabetics, both in humans and in animal models.

**Conclusion**

There is clear evidence that autophagy is crucial to the maintenance of normal pancreatic β-cells, influences diabetic nephropathy, and is important in the progression of obesity-induced insulin resistance to type 2 diabetes. The autophagic process may present a novel therapeutic target for future treatments and prevention of type 2 diabetes. While several pharmacological upregulators of autophagy currently exist, the safety and efficacy of such agents in type 2 diabetic humans is unknown. Exercise may present a novel approach to autophagy upregulation in individuals with impaired fasting glucose and type 2 diabetics; however, studies examining the effects of exercise on autophagy in these disorders are lacking. A much greater understanding of the relationship between exercise, autophagy, insulin resistance and diabetes is required to determine the efficacy of exercise-induced autophagic therapies.
References


CHAPTER 3
RESEARCH MANUSCRIPT

This chapter presents a research manuscript, entitled “The effect of acute aerobic exercise and rapamycin treatment on autophagy and the heat shock response in peripheral blood mononuclear cells of prediabetics”. This manuscript will be submitted to the “Canadian Journal of Diabetes”. It is authored by James J. McCormick, Karol Dokladny, Kelli E. King, Fabiano Amorim, Len R. Kravitz, and Christine M. Mermier. The manuscript follows the formatting and style guidelines of the journal. References and figures are provided at the end of the chapter.
Title: The effect of acute aerobic exercise and rapamycin treatment on autophagy and the heat shock response in peripheral blood mononuclear cells of prediabetics

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Running Head: Autophagy is impaired in prediabetic PBMCs

Keywords: LC3, HSP72, MAP1LC3B, p62/SQSTM1, HSPA1A, prediabetes, autophagy, rapamycin
ABSTRACT

Objectives: Recently, a malfunction of the autophagic pathway has been implicated with impaired glucose metabolism and progression from prediabetes to type 2 diabetes. Further, an interconnection has been suggested between the autophagy and heat shock protein (HSP) systems. The aims of the present study were to investigate the effects of exercise and rapamycin treatment (RAPA) on the autophagic process and HSP in peripheral blood mononuclear cells (PBMCs) from prediabetics compared to controls.

Methods: Two groups matched for age and sex served as subjects and consisted of six prediabetic (42.4±11.7) and six controls (44.4±11.9). Subjects exercised at 50% of VO_{2max} for 60 min with 5 min of rest dispersed every 20 min. PBMCs were isolated pre-exercise, immediately post-exercise, and 4hr after exercise recovery. Additional PBMCs were either exposed to bafilomycin (BAF), rapamycin with bafilomycin (RAPA), or no treatment (NT) with vehicle (dimethylsulfoxide). Proteins and mRNA were analyzed via western blot and quantitative real-time polymerase chain reaction.

Results: Exercise increased autophagy immediately post-exercise and recovered 4hr after exercise in control subjects but not in prediabetics. Prediabetic and control subjects both increased autophagy following RAPA treatment; however, a significantly impaired response was observed in prediabetics when compared to controls. No significant differences in HSP72 response was observed.

Conclusions: Our results indicate an impairment in autophagic flux but not HSP in PBMCs from prediabetics when compared to controls in response to both exercise and RAPA treatment. Future methods of autophagic upregulation should be investigated to spare malfunctions in autophagy in prediabetics.
Introduction

Type 2 diabetes is a disease with increasing prevalence that has become a global health problem that affects people of all socioeconomic classes. According to the International Diabetes Foundation Atlas, the estimated diabetes prevalence in 2017 was 425 million, with over 641 million people predicted to be living with type 2 diabetes worldwide by the year 2040 [1]. It is well established that type 2 diabetes is associated with increased risk of developing microvascular disorders including nephropathy, neuropathy, and retinopathy [2]. Further, individuals with type 2 diabetes have increased morbidity and mortality due to increased risk of cardiovascular disorders such as coronary artery disease, hypertension, and stroke [3, 4]. However, prior to the development of type 2 diabetes, a period of prediabetic state occurs which may be characterized by impaired glucose and insulin tolerance, mild to moderate obesity, and fluctuations in normoglycemic and hyperglycemic states [5].

Exercise is known to play a fundamental role in the prevention and treatment of type 2 diabetes, in part due to improvements in insulin sensitivity and increased maximal oxygen consumption (VO_{2\text{max}}) [6]. Additionally, exercise-induced adaptations include an increased abundance of proteins involved with insulin signaling (such as insulin receptor substrate 1) and glucose metabolism important in cellular homeostasis [7, 8]. However, these metabolic adaptations have been reported to be impaired in type 2 diabetics and patients with prediabetes, indicating a potential resistance to the beneficial effects of exercise [9].

Macroautophagy (herein referred to as autophagy) is a catabolic cellular process, which involves the degradation of protein aggregates and damaged organelles, and is crucial in maintaining cellular homeostasis [10]. Stimulation of autophagy has been shown to be crucial in the development of cellular adaptations to exercise [11]. However, disruption of autophagy in
mice has been shown to impair endurance exercise performance and glucose metabolism during an acute exercise bout [12]. Further, impaired autophagic functioning may contribute to the development of prediabetes and type 2 diabetes through the development of insulin-resistance [13] and other comorbidities including neurodegenerative disorders [14, 15], cardiomyopathy [16], and cancer [17]. While the beneficial effects of exercise in prediabetes and type 2 diabetes have been well established [18], little is known about the effect of exercise in prediabetics and type 2 diabetics. It has recently been demonstrated that an acute bout of endurance exercise (1-2h) is sufficient to stimulate autophagic signaling in skeletal muscle of young healthy adult males [19, 20]. Recent evidence suggests that the acute autophagic response may be intact in human skeletal muscle of type 2 diabetics in response to 60 min of cycling exercise at 70% of VO$_{2\text{max}}$ [21]. This may be due to an autophagic adaptation to chronic hyperglycemic conditions, however it is unknown if prediabetics exhibit this adaptive response [22]. Thus, the autophagic response to exercise in prediabetic individuals remains to be established.

Heat shock proteins are a family of proteins named based on their molecular weight and decreased expression may play a role in the development of type 2 diabetes. It is thought that heat shock protein 72 (HSP72) expression may interact directly with glucose transporter type 4 (GLUT4) translocation, thereby modulating insulin sensitivity [23]. Further, there is evidence that HSPs may interact with autophagic signaling in young healthy males [24, 25]; however, this has not been established in prediabetic individuals.

The aim of the present study was to investigate the response of autophagy and HSP in response to an acute bout of exercise and subsequent recovery in peripheral blood mononuclear cells (PBMC) of individuals with prediabetes compared to age and sex matched controls. To
further elucidate the mechanism of autophagic modulation, we treated PBMCs from prediabetics with known autophagy inducer rapamycin.

Methods

Human subjects

The study was approved by the University of New Mexico’s (UNM) Human Research Review Committee (HRPO-14-200) and all subjects provided written informed consent. Twelve adult participants (6 males, 6 females) between the ages of 27-61 years (yr) were recruited who were free of known illness or disease. Subjects falling outside of the age ranges, currently taking glucose lowering or statin medications, or experiencing an acute illness were excluded. Subjects were categorized into the prediabetic group, determined via a hemoglobin A1C (HbA1C) blood test value greater than 5.7% and less than 6.4% [26]. Control subjects were age and sex matched with the prediabetic group and categorized by having an HbA1C value less than 5.7%.

Maximal exercise test

Prior to the experimental trial, all subjects were asked to refrain from rigorous exercise for at least 48 hours, to abstain from alcohol for at least 24 hours, and caffeine for 12 hours. A small blood sample was drawn for the measurement of HbA1c. Waist circumference, subject height and weight were recorded, and body fat percentage was estimated using a bioelectrical impedance analyzer (Omron-HBF306, Bannockburn, IL) immediately prior to the measurement of maximal oxygen consumption (VO$_{2\max}$). All VO$_{2\max}$ tests were conducted on a cycle ergometer (Lode Excalibur, Gronigen, The Netherlands) and included a 5-minute self-selected warm-up. A maximal exercise test was performed using a ramp protocol in which power was increased every 60s at an individualized rate, based on subjects’ size, exercise and health history to induce fatigue within 8 to 12 min. Expired air was analyzed breath-by-breath during the
exercise test using a metabolic cart (Parvomedics, TrueOne 2400, Sandy, UT) and calibrated as recommended by the manufacturer. Heart rate (HR) was recorded using a HR transmitter strap (Polar, T-31, Lake Success, NY) integrated to the metabolic cart.

**Aerobic exercise session**

Subjects were asked to refrain from vigorous exercise for at least 48 hours and to abstain from caffeine and alcoholic beverages for at least 24 hours before the experimental trial. The subjects ingested a standardized breakfast (240 kcal of total energy intake, 62% carbohydrate, 17% protein, and 21% fat) 2 hours before the start of exercise. Once they arrived at the lab they rested for 20 min in a seated position before the initial blood draw and exercise session.

The exercise session consisted of a 5-min warm-up while cycling at an intensity corresponding to 20% of the peak power achieved in the VO$_2$max test. The workload was then increased to correspond with 50% of VO$_2$max, as confirmed by the metabolic cart. Subjects continued to exercise for 60-min (three sets of 20-min separated by 5 min intervals of passive rest). Water was provided ad libitum throughout the exercise trials.

**PBMC Collection**

Venous blood was collected via venipuncture from an antecubital vein prior to the aerobic exercise session and following 20-min in a seated position. Venous blood was also drawn immediately after the 60-min exercise session and 4h after termination of the exercise session. Peripheral blood mononuclear cells were immediately isolated from whole blood and frozen at -80°C until analyzed.

**Cell Experiments**

Immediately prior to the aerobic exercise session, peripheral blood mononuclear cells were isolated from whole blood suspended in histopaque (Histopaque-1077, Sigma-Aldrich) and
incubated at 37° C for a period of 24 hours in cell culture medium containing MegaCell RPMI-1640 (Sigma-Aldrich; F4135), 1% L-glutamine (ThermoFisher; 25030081), 1% Penicillin-Streptomycin (ThermoFisher; 15140122), and 5% fetal bovine serum (Sigma-Aldrich; F4135). Peripheral blood mononuclear cells were then transferred to cell culture plates and were either treated with dimethyl sulfoxide vehicle (DMSO; Sigma-Aldrich, 472301), bafilomycin A1 in DMSO (Invivogen, tlr-bafl)(100 nM), or bafilomycin (BAF) and rapamycin (Rapa) in DMSO (Invivogen, tlr-rap) (0.5 nM) for 2 hours in 37° C. Bafilomycin was used to block the formation of the autophagolysosome preventing LC3-II degradation, thus enabling the quantification of autophagy via LC3-II accumulation [27, 28]. Treatment with Rapa simulated starvation-induced autophagy via upstream inhibition of mTOR. Cells were immediately harvested and stored at -80° C until analyzed.

**Immunoblot analysis**

Cells were lysed in a modified RIPA buffer (Tris-HCl 8.0 pH; Invitrogen, 15568-025); 0.5M EDTA (Invitrogen, 15568-020); 1.5M NaCl (Sigma-Aldrich, S9888) 1% Triton X 100 (Sigma-Aldrich, X100); and freshly added protease (ThermoScientific, 78430) and phosphatase (ThermoScientific, 7842) inhibitors. HSP72, LC3, SQSTM1/p62, and β-actin were resolved by electrophoresis in a 12% polyacrylamide gel (Bio-Rad, 456-144). Proteins were transferred to cellulose membranes (Bio-Rad, 162-0094) then blocked in tris buffered saline (150 mM NaCl, pH 8.0) containing 0.2% polysorbate (Tween 20; Bio-Rad, 170-6531) detergent and 5% powdered milk (Bio-Rad, 170-6404). Membranes were then incubated in tris-buffered saline containing 0.2% polysorbate detergent and 5% bovine serum albumin (Sigma-Aldrich, A9418) with primary antibodies including: LC3 (Sigma-Aldrich, L7543), SQSTM1/p62 (abcam, ab56416), HSP72 (Enzo, P08107) and β-actin (Sigma-Aldrich, A5441). Primary antibodies were
detected by horseradish peroxidase-labeled secondary antibody (Goat anti-rabbit; Cell Signaling, 7074s; Goat anti-mouse; Cell Signaling, 70076s) binding, which was detected using Santa Cruz Western blotting luminol reagents (Santa Cruz Biotechnology, Santa Cruz, CA) and imaged with the ChemiDoc Touch (Bio-Rad, Touch, CA).

**Quantitative RT-PCR**

Total RNA was isolated from human PBMCs using the QIAshredder (Qiagen, 79654) and RNeasy Mini Kit (Qiagen, 74104), then reverse transcribed using the cDNA Reverse Transcription Kit (Roche, 64869866001). cDNAs were amplified in a StepOnePlus Lightcycler (Applied Biosystems, Grand Island, NY, USA) using the following amplification conditions: PCR initial activation step, 95 ºC for 10 min; two-step cycling, 40 cycles of denaturation, 95 ºC for 15 s; combined annealing/extension, 60 ºC for 1 min. The sequence specific primers used in the reactions were: for MAP1LC3B (Applied Biosystems, Hs00792944_s1): forward primer, 5’-AGCAGCATCCAAACCAAAATC-3’ and reverse primer 5’-CTGTGTCCGTTCACCAACAG-3’; for SQSTM1/p62 (Applied Biosystems, Hs01061917_g1): forward primer, 5’-cacctgtctgagggcttctc-3’; reverse primer, 5’-agtttcctggtggacccatt-3’; for HSPA1A (Applied Biosystems, Hs00359163_s1): forward primer 5’-TGGACTGTCTCTCACTCTTGGC-3’ and reverse primer: 5’-TCCGGAGAGTTCTGGGATTGTA-3’ were used as previously published [29, 30]. Gene expression was normalized to reference gene β-actin (internal control gene) (Applied Biosystems, 4326315E). Relative quantification of the MAP1LC3B and HSPA1A genes were calculated relative to β-actin and the mean fold change in expression of the MAP1LC3B and HSPA1A genes were calculated using $2^{-\Delta\Delta CT}$ method.
**Statistical Analysis**

All data are presented as mean ± SD. Comparisons between and within groups were performed by a repeated measures two-way analysis of variation (ANOVA), followed by a Tukey’s post-test, using SPSS software (IBM, version 19). Single statistical comparisons of clinical and metabolic characteristics between prediabetics and controls were performed with a two-tailed Student’s t test. All reported levels of significance are indicated as $p < 0.05$.

**Results**

**Clinical and metabolic characteristics**

Participants with prediabetes had significantly higher HbA1c levels ($p = 0.001$) than control participants but showed a similar VO$_{2\text{max}}$ ($p = 0.36$) indicating minimal differences in cardiorespiratory fitness between groups. Further, no differences were observed in BMI ($p = 0.47$), body fat percentage ($p = 0.56$), or waist circumference ($p = 0.49$) between prediabetic and control participants (Table 1).

**The effect of exercise on markers of autophagy and the heat shock response**

The protein abundance of important autophagy markers was examined between prediabetic and control PBMCs. LC3-II and p62/SQSTM1 are degraded with autophagy in most cells indicating increased autophagy. No differences were observed in response to exercise between groups; despite a significant increase in LC3-II protein accumulation ($p = 0.03$) following 4h recovery in the control group. Lipidation of LC3-I to LC3-II is a commonly used indicator of autophagosome abundance [31]. A significant decrease in LC3-II/I ratio was observed immediately post-exercise in the control group ($p = 0.04$), but not in in the prediabetic group, which was restored following 4h recovery (Figure 1c). A comparison of the basal levels
of autophagy between groups showed no differences for LC3-II or p62/SQSTM1 protein abundance. However, the prediabetic group showed a significantly higher LC3-II/I protein ratio ($p = 0.01$) when compared to control basal conditions (Figure 5c).

The mRNA levels of MAP1LC3B and p62/SQSTM1 were also examined. No significant differences were observed under any condition; however, the diabetic group showed a non-significant lower expression of all genes compared to controls under all conditions.

No significant differences were observed in HSP72 protein expression under any condition for either group. Further, no differences were observed in HSPA1A mRNA expression following exercise in prediabetics or controls. No differences were observed in basal HSP72 levels between groups.

The effect of rapamycin treatment on markers of autophagy and the heat shock response

Rapamycin treatment was used to block mTORC1, which is a known autophagy inducer [27]. Additionally, bafilomycin was used to block autophagic degradation. A significant increase in LC3-II protein was observed following BAF treatment in the control group ($p < 0.01$) but not the prediabetic group (Figure 3a). Following RAPA treatment, a significant increase in LC3-II protein abundance was observed in both the prediabetic ($p = 0.04$) and control groups ($p = 0.01$); however, the increase in the control group was significantly higher than the prediabetic group ($p = 0.02$) (Figure 3a). A significant reduction in p62/SQSTM1 protein abundance compared to NT was observed following BAF treatment in the prediabetic group ($p = 0.01$) but not the control group, with a significant reduction of p62/SQSTM1 in both groups ($p < 0.01$ and $p = 0.03$ for the prediabetic and control groups, respectively) compared to NT following RAPA treatment (Figure 3b). A significant increase in LC3-II/I ratio was observed under BAF and RAPA conditions for the control group ($p < 0.01$ and $p = 0.03$, respectively) but not the prediabetic
group (Figure 3c). No significant differences were observed in response to BAF or RAPA conditions in HSP70 protein abundance.

When examining differences in gene expression, no changes were observed in MAP1LC3B expression between groups under BAF or RAPA conditions. A significant increase was observed in p62/SQSTM1 expression following RAPA treatment compared to NT was observed in both controls ($p = 0.02$) and prediabetics ($p < 0.01$) (Figure 4b). Further, no significant differences were observed for HSPA1A between either group.

**Discussion**

In the present study, we examined the impact of an acute bout of moderate intensity (50% $\text{VO}_{2\text{max}}$) endurance exercise on markers of autophagy and the heat shock response in PBMCs of individuals with prediabetes and age-sex matched controls. To further understand differences in autophagy and heat shock responses between prediabetics and controls, we treated PBMCs from the same prediabetics and controls with RAPA. Our findings suggest a minor impairment of the autophagic response in prediabetics immediately post-exercise, with a recovery of autophagic flux within 4h recovery in control individuals. Further, we found a blunted autophagic response in the prediabetic group compared to controls when exposed to RAPA treatment. We found no differences in the heat shock response between prediabetics and controls under exercise or RAPA treatment conditions.

In response to acute endurance exercise, there was a decrease in LC3-II/I ratio immediately following exercise cessation in the control group but not the prediabetic group indicating greater autophagic flux [27]; however, levels were restored within 4h of recovery. This is further evidenced by the significant increase in LC3-II protein accumulation observed in
the control group 4h post exercise, but not in the prediabetic group. The observed drop in LC3-II/I protein ratio in the control group is similar to previous findings that found a drop in LC3-II/I protein ratio immediately after acute endurance exercise in human skeletal muscle of young, healthy males [19, 21]. However, previous findings by Kruse et al. [21] did not show any differences in autophagic initiation between type 2 diabetics and controls following acute endurance exercise in human skeletal muscle. As autophagy measurements were only made in PBMCs in the present study, these differences may be explained by the highly tissue specific nature of the autophagic response to stress [32]. Previous findings have reported significantly reduced MAP1LC3B mRNA expression following exercise and 3h recovery in type 2 diabetics when compared to controls and no differences in p62/SQSTM1 in skeletal muscle [21]. Our present findings show no differences in MAP1LC3B or p6/SQSTM1 mRNA expression, suggesting transcriptional regulation of autophagy remains intact in prediabetics.

Increased levels of LC3-II and decreased levels of p62/SQSTM1 are typically indicative of increased autophagic flux [27]. We found no differences in p62/SQSTM1 protein abundance in response to exercise in either group. These results are similar to previous findings which showed no difference in p62/SQSTM1 protein regulation following acute exercise in healthy, young males [19, 20] or in type 2 diabetic patients [21]. This may suggest an inhibition of autophagosome formation independent of cargo protein p62/SQSTM1. Previous findings in type 2 diabetics have shown p62/SQSTM1 accumulation [22], or no change [27] following strong levels of autophagy induction in skeletal muscle suggesting p62/SQSTM1 responses may be tissue specific. It is plausible that the lack of degradation in p62/SQSTM1 in the present study was independent of changes in autophagic flux. This may be through mechanisms involving tumor protein p53 inducible nuclear protein 2 (TP53INP2) which may act as an autophagic cargo
protein independent of p62/SQSTM1 [33]. Taken together, our findings support the interpretation that LC3-II/I reduction in the absence of p62/SQSTM1 regulation may indicate increased autophagic turnover (lysosomal degradation) following exercise, as observed in our control participants.

Autophagy induction via RAPA treatment induced much more pronounced differences in autophagic activation between control and prediabetic patients. Bafilomycin was used to block autophagolysosome formation, thus enabling the accumulation of LC3-II protein with increased autophagy. Following RAPA treatment, LC3-II protein was significantly higher in control PBMCs than in those of prediabetics, which is a key marker of autophagosome formation [27]. This was accompanied by a significant increase in LC3-II/I ratio in controls indicating increased autophagic flux that was not observed in prediabetic individuals. The findings in controls are similar to those found by Dokladny, et al. [24], where a marked increase in LC3-II abundance was observed following RAPA treatment in A549 cells. This is unsurprising, as RAPA treatment blocks mTORC1 in a similar manner to starvation, a potent inducer of autophagy [34]. There is increasing evidence supporting the hypothesis of dysregulated autophagy in obesity and type 2 diabetes [22]. The present findings suggest a reduction in autophagosome formation in response to RAPA treatment in prediabetics, further supporting autophagic dysregulation found in type 2 diabetes [22]. While the mechanism of autophagy reduction in prediabetics is presently unknown, impaired insulin signaling, mitochondrial dysfunction and altered protein metabolism, are all known characteristics of prediabetes and type 2 diabetes [8, 35, 36], which may contribute to disruptions in autophagy [37].

Interestingly, despite an impaired response in LC3-II protein abundance and LC3-II/I ratio, a significant decrease in p62/SQSTM1 was observed in both prediabetic individuals and
controls. This may suggest an inhibition of autophagosome formation independent of cargo protein p62/SQSTM1 in prediabetics indicating a preservation of the selective autophagy response [38]. Previous research has reported no changes in p62/SQSTM1 accumulation following insulin treatment in muscle biopsies of young, healthy individuals [39]. As RAPA directly inhibits mTORC1 downstream of the insulin-signaling pathway, it is possible that observed changes in p62/SQSTM1 were independent of autophagic flux. However, as transcription of p62/SQSTM1 increased in both controls and prediabetics in the presence of decreased protein abundance, it is more likely indicative of an increased autophagic response rather than an independent mechanism as described by others [21, 22]. This is an important response, as it enables the autophagic process to sort and remove vacuolar enzymes and aggregate-prone proteins and unwanted organelles that may contribute to the development of insulin-resistance [40, 41].

The heat shock response represents a protein management system in which HSPs prevent protein aggregation and facilitate refolding [42]. Under stressful conditions, increased HSP72 protein expression allows cells to survive harsh conditions and restore cellular homeostasis through maintenance of proper protein structure [43, 44]. Our present findings indicate no significant differences between HSP72 protein expression or HSPA1A mRNA levels in response to acute endurance exercise or RAPA treatment. Our RAPA treatment findings are in agreement with past research, indicating minimal acute HSP72 protein expression following autophagy induction in response to RAPA treatment [24]. Autophagy functioning to maintain energy and protein homeostasis has been shown to be under the regulatory control of HSP72 [24]. Previous research has shown HSP72 upregulation following 4h recovery post-exercise; however, this was under more strenuous conditions (70-80% VO_{2max}) than in the present study [24]. A more intense
exercise stimulus and HSP72 measurements exceeding 4h may be required to fully understand the relationship between autophagy and the heat shock response in prediabetic individuals.

The limitations of the present study include the small sample size and lack of additional markers of autophagy regulation and upstream signaling. Further, we have no data on the course of autophagy and HSP72 regulation beyond 4h recovery post-exercise. We identified quantitatively small, yet significant changes in autophagy responses which are consistent with findings presented in other studies [21, 24] which may reflect the highly conserved nature of the autophagic response under physiologic conditions, as large changes in this process may have damaging cellular consequences. Lastly, our findings are limited to PBMCs, which may not coincide with responses observed in skeletal muscle.

In summary, our findings demonstrate a small inhibition in the autophagic response to exercise in prediabetic individuals. Further, we show autophagic inhibition following RAPA treatment in PBMCs, indicating a reduced capacity to upregulate autophagy under mTORC1-limiting conditions in prediabetics. Future studies are required using different exercise intensities and measurements beyond 4h recovery to gain a more complete understanding of the relationship between exercise, autophagy, and the heat shock response in prediabetes.
References


Table 1 Clinical and metabolic characteristics at study entry
Data are presented as means ± SD. * P < 0.05 compared with control.

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<td>Age (years)</td>
<td>42.4 ± 11.7</td>
<td>44.4 ± 11.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.7 ± 2.5</td>
<td>24.2 ± 5.6</td>
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<tr>
<td>Body Fat (%)</td>
<td>23.5 ± 7.2</td>
<td>25.6 ± 5.9</td>
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<tr>
<td>Waist Circumference (cm)</td>
<td>80.8 ± 3.5</td>
<td>83.9 ± 15.3</td>
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<tr>
<td>VO₂max (ml/kg/min)</td>
<td>38.6 ± 9.1</td>
<td>36.8 ± 6.8</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.3 ± 0.1</td>
<td>6.0 ± 0.2*</td>
</tr>
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Figure 1 Exercise-mediated regulation of protein markers of autophagy and heat shock response
Protein content of (a) LC3-II, (b) p62/SQSTM1, (c) LC3-II/I, and (d) HSP72 in peripheral blood mononuclear cells of individuals with prediabetes (black bars) and control individuals (white bars) before (pre-ex), immediately after exercise (IPE), and 4 h into recovery (4h rec). (e) Representative blots (P, pre-exercise; E, post-exercise; R, 4h into recovery). Data are means ± S.E.M.; * indicates p < 0.05 compared with respective control; # indicates p < 0.05 compared with pre-exercise.
Figure 2 Exercise-mediated transcriptional regulation of autophagy and heat shock response
mRNA expression of genes of (a) MAP1LC3B, (b) p62/SQSTM1, and (c) HSPA1A in peripheral blood mononuclear cells of patients with prediabetes (black bars) and control individuals (white bars) before (pre-ex), immediately after exercise (IPE), and 4 h intro recovery (4h rec). Data are means ± S.E.M.
Figure 3 Rapamycin-mediated regulation of protein markers of autophagy and heat shock response

Protein content of (a) LC3-II, (b) p62/SQSTM1, (c) LC3-II/I, and (d) HSP72 in peripheral blood mononuclear cells of individuals with prediabetes (black bars) and control individuals (white bars) with no-treatment (NT), bafilomycin treatment (BAF), and bafilomycin with rapamycin treatment (BAF + RAPA). (e) Representative blots (N, no treatment; B, bafilomycin treatment; R, bafilomycin with rapamycin treatment). Data are means ± S.E.M.; * indicates $p < 0.05$ compared with respective control; # indicates $p < 0.05$ compared with NT; $\phi$ indicates $p < 0.05$ compared with BAF.
Figure 4 Rapamycin-mediated transcriptional regulation of markers of autophagy and heat shock response

mRNA expression of genes for (a) MAP1LC3B, (b) p62/SQSTM1, and (c) HSPA1A in peripheral blood mononuclear cells of individuals with prediabetes (black bars) and control individuals (white bars) with no-treatment (NT), bafilomycin treatment (BAF), and bafilomycin with rapamycin treatment (BAF + RAPA). Data are means ± S.E.M.; # indicates $p < 0.05$ compared with NT. 
CHAPTER 4

Abstract
This chapter presents a summary, conclusions, and suggestions for future research. Additionally, data is included that was not included within the research manuscript entitled, “The effect of acute aerobic exercise and rapamycin treatment on autophagy and the heat shock response in peripheral blood mononuclear cells of prediabetics” as this was listed as extra data that was collected but did not fit within the final publication. These data include a version of the tabled subject characteristics with non-significant p-values and basal levels of autophagy.

Implications for Autophagy in the Prevention and Treatment of Metabolic Disease

Autophagy is a crucial cellular mechanism that has gained much attention in recent years for its role in several metabolic and degenerative diseases, including diabetes, Huntington’s disease, Alzheimer’s disease, and Parkinson’s disease. Further, a decline in autophagic activity may have a causative role in the functional deterioration of biological systems during aging. Despite the known importance of autophagic functioning in disease prevention, very little is known about methods of safe autophagic stimulation in humans and mechanisms leading to the impairment of autophagy and its connection to disease progression. Currently, research investigating the role of autophagy in prediabetes and type 2 diabetes in humans is in its infancy. To date, only one study has been published examining the effects of acute endurance exercise in type 2 diabetics. Our study represents the first study to examine the effect of acute endurance exercise in peripheral blood mononuclear cells (PBMC) of prediabetics. Despite our current contribution to the body of growing autophagy research, much more research needs to be conducted to understand the role of autophagy in various metabolic diseases, such as diabetes.
Further, there is very little understanding on the effect of chronic exercise on autophagy activation in those at risk for metabolic disease or who currently have metabolic disease. Specifically, future research is needed to elucidate the impact of various exercise intensities and durations to determine the most effective combination to stimulate autophagy.

Our study is also the first to examine the effect of mammalian target of rapamycin (mTOR) inhibition via rapamycin treatment on autophagy in cells of prediabetics. While rapamycin is a potent activator of autophagy in healthy cells, we demonstrate an impairment in autophagic activation in PBMCs of prediabetics. Additionally, it is yet to be determined whether rapamycin is a safe a treatment for humans, as whole body mTOR inhibition has detrimental effects. Rapamycin has been used for decades to suppress the immune system in transplant patients. Inhibiting mTOR with rapamycin has other, systemic side-effects in humans that are not fully understood, including insulin resistance. Thus, future investigation is necessary to determine other safe and effective activators of autophagy in cells of prediabetics and in other tissues in human subjects.
APPENDICES

A. Combined HIPAA/Informed Consent
B. Flyer
C. Recruitment Email
D. Health History Questionnaire
E. Session 1 Data Sheet
F. Session 2 Data Sheet
G. Supplemental Figure 1
H. Supplemental Figure 2
The University of New Mexico
Combined Consent/HIPAA Authorization to Participate in Research
[05/19/2014]

Introduction

You are being asked to participate in a research study that is being done by Christine Mermier, Ph.D., who is the Principal Investigator and her associates, from the Department of Health, Exercise and Sports Sciences and Department of Internal Medicine. This research is studying the effects of a single moderate intensity aerobic exercise session on response markers of autophagy in those with and without glucose intolerance. Autophagy is a beneficial cell survival process that is induced during exercise.

You are being asked to participate in this study because you either have normal fasting glucose or impaired fasting glucose, but are not taking any medicine that is used to lower your blood sugar or to lower your cholesterol. You should be otherwise healthy enough to exercise on a cycle for 20 minutes at a time. Twenty people will take part in this study at the University of New Mexico.

The University of New Mexico’s College of Education is funding this study.

This form will explain the research study, and will also explain the possible risks as well as the possible benefits to you. We encourage you to talk with your family and friends before you decide to take part in this research study. If you have any questions, please ask one of the study investigators.

What will happen if I decide to participate?

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

Questionnaires and consent: You will be asked to read and sign this consent form if you agree to be in the study. We will ask you to fill out questionnaires regarding your exercise history and your health history. If you have not had your blood sugar (hemoglobin A1C) tested recently (within 6 months), we will ask to draw your blood for this test. Completion of all of these forms (and the blood draw if needed) should take no more than 30 minutes. Results of the blood sugar will determine, in part, if you can participate in the study.

Exercise testing sessions:

Session #1: During your first session you will come to the UNM exercise physiology lab in Johnson Center, Room B143. We will measure your height, weight, waist circumference, and body fat with a handheld device called a bioelectrical impedance analyzer.

You will then have ten electrodes (sticky tabs) placed on your chest to monitor your heart rhythms. You will be fitted to be comfortable on our stationary cycle that will be used for your maximal exercise test. Once you are comfortably seated on the bike, we will ask you to put on
a device to help us measure your oxygen consumption and will be worn throughout this test. The exercise test will last approximately 8 to 12 minutes. During the exercise test, we will be continuously monitoring your heart rate and rhythms, and will be asking you how you feel on a scale of 6-20, with 6 being easy and 20 being maximal effort. The oxygen consumption collection device has a rubber part that goes in your mouth like a snorkel, and a clip that goes on your nose. You will be breathing normal air. Though the device can be a bit uncomfortable, you will be able to breathe normally, but only through your mouth. You will then be asked to start pedaling the bike, and to pedal continuously until you can no longer maintain at least 50 revolutions per minute. The test will start out with easy pedaling, but gets increasingly harder until you can no longer continue. We will be encouraging you to keep going as long as you can, but you are free to stop the test at any time. We will stop the test if we see any abnormalities of your heart. After you finish the test, the mouthpiece and nose clip will be immediately removed and you will pedal slowly to cool down as long as you need and until your heart rate returns to near baseline. After removal of the electrodes, we will schedule you for your next test session which will occur at least 48 hours after the maximal exercise test. You will be given an energy bar to eat two before your next appointment and then you will be allowed to leave. The total time for this session will be about 1.5 hours.

Session #2: You will come to the same location as you did in session #1. You will eat only the food that was given to you in the first session, and you may drink as much water as you like. However, we will ask you to refrain from any other beverages or food, especially anything containing caffeine. We will also ask you to not exercise or drink alcohol for 24 hours before the test.

After 10 minutes of seated rest, a small amount (see below for exact amounts) of blood will be drawn from a vein in your arm. You will then be seated on the bike and allowed to warm up for five minutes before the workload is increased to an intensity equal to 50% of the oxygen consumption (VO$_2$) that you achieved during you maximal exercise test. You will be asked to complete 60 minutes of exercise (three sets of 20 minutes separated by 5 minutes of rest). At about the 10 minute point of each 20 minute bout, we will measure your VO$_2$ for 2 minutes with the same mouthpiece and nose clip device as explained above. The device will only stay on for those two minutes. After you complete the 60 minutes of exercise, you will sit down for 10 minutes, and then we will again draw your blood. You will be asked for one more blood sample four hours after completion of the exercise. You may either stay in the lab and read or work on the computer, or you may leave and come back 15 minutes before your scheduled blood draw. We will ask you to drink only water and to refrain from eating and exercise until the last blood sample is collected. The total time for this session (including the time between the second and third blood draw) will be six hours. The final blood draw will end your participation in our study.

Blood collection: We will ask you to allow us to collect blood three separate times for us to assess your autophagy markers. The pre-exercise blood draw will be 30 ml (2 tablespoons), and the post-exercise and 4 hours post-exercise will each be 15 ml (1 tablespoon). If you do not have a current hemoglobin A1C lab test (no older than six months), this may require an additional blood draw of less than a teaspoon (3 ml). The total amount of blood drawn over the study will be no more than 60 ml (4 tablespoons). Your blood will be labeled only with a special number which will not be linked to your name. We will store the blood until we are ready to analyze it, and we may keep these samples for up to two years.
**How long will I be in this study?**

Participation in this study will take a total of 8 hours over a period of 2-3 sessions. The first session may not be required if you have a current lab test for your blood sugar (HbA1C). If you have a current blood test, the questionnaires may be done before the first exercise test session. Regardless if you come to the lab twice or three times, the total time commitment will still be approximately 8 hours.

**What are the risks or side effects of being in this study?**

Risks associated with maximal exercise testing may include the following: brief feelings of nausea, lightheadedness, muscle cramps, or dizziness during or after completion of exercise. According to the American College of Sports Medicine, the risk of a cardiac event in normal healthy individuals during a maximal exercise test is minimal, 0.0006% (6 in 10,000). Experienced personnel will be monitoring your heart rate and rhythms throughout the test and will stop the test if abnormalities are detected. You will be allowed to stop if you don’t feel well or do not want to continue. The exercise sessions could cause muscle soreness that could last several days. The device used to measure oxygen consumption may be uncomfortable, however, we will limit the amount of time you will be required to wear the device.

Drawing blood may cause temporary pain and discomfort from the needle stick, occasional bruising, sweating, feeling faint or lightheaded, and in rare cases, infection. Experienced phlebotomists, a physician assistant, or physician will draw your blood.

You also may be uncomfortable having to refrain from having any caffeine, smoking or drinking alcohol or eating any food other than what is provided to you before the second exercise testing session. This study requires several hours of your time on the second exercise test visit and you may be bored or inconvenienced waiting for the final blood draw four hours after the exercise. There are risks of stress, emotional distress, inconvenience and possible loss of privacy and confidentiality associated with participating in a research study.

**What are the benefits to being in this study?**

There will be no direct benefit to you from participating in this study. However, it is hoped that information gained from this study will help us understand the relationship of autophagy activation and glucose control through exercise in humans. We will also give you the results of your body fat and VO$_{2\text{max}}$, and HbA1C (if applicable) tests, which may help you understand some important variables associated with your health.

**What other choices do I have if I do not want to be in this study?**

You have the option not to take part in this study. There will be no penalties involved if you choose not to take part in this study.
How will my information be kept confidential?

We will take measures to protect the security of all your personal information, but we cannot guarantee confidentiality of all study data.

Information contained in your study records is used by study staff and, in some cases it will be shared with the sponsor of the study. The University of New Mexico Institutional Review Board (IRB) that oversees human subject research and/or other entities may be permitted to access your records. There may be times when we are required by law to share your information. Your name will not be used in any published reports about this study.

Information and blood samples collected as part of the study will be labeled with your study number only; Information (without your name) will be entered into a computer database and hard copies of the data sheets will be placed in a locked file cabinet in the Principal Investigator’s office. Dr. Mermier and her associates will have access to your study information. Data will be stored for up to five years, and then will be destroyed.

What are the costs of taking part in this study?

There will be no cost to you to take part in the study other than the possible cost of parking at the university during the required sessions.

Will I be paid for taking part in this study?

In return for your time, parking fees, and the inconvenience of participating in this study, you will be paid $25 in the form of a gift card for each of the exercise testing sessions, for a total of $50 in gift cards. If you do not complete the study, you will be given a $25 gift card for each exercise test session you complete.

Compensation is considered taxable income. Amounts of $600 or more will be reported by UNM to the Internal Revenue Service (IRS).

How will I know if you learn something new that may change my mind about participating?

You will be informed of any significant new findings that become available during the course of the study, such as changes in the risks or benefits resulting from participating in the research.

Can I stop being in the study once I begin?

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 without affecting your future health care or other services to which you are entitled. If we feel it would be unsafe for you to continue your participation, we will withdraw you from the study. You may request that
your data not be included in the study, by writing to the principal investigator at the following address:

Christine Mermier, Ph.D
1 University of New Mexico
MSC04 2610
Albuquerque, New Mexico 87131

**HIPAA Authorization for Use and Disclosure of Your Protected Health Information (HIPAA)**

As part of this study, we will be collecting health information about you and sharing it with others. This information is “protected” because it is identifiable or “linked” to you.

**Protected Health Information (PHI)**

By signing this Consent Document, you are allowing the investigators and other authorized personnel to use your protected health information for the purposes of this study. This information may include: a test for blood sugar (HbA1C), height, weight, age, %body fat, results of VO_{2max} testing, medical history, and waist circumference. You will be asked to let us know if you are taking certain medications which may make you ineligible to participate.

In addition to researchers and staff at UNM and other groups listed in this form, there is a chance that your health information may be shared (re-disclosed) outside of the research study and no longer be protected by federal privacy laws. Examples of this include disclosures for law enforcement, judicial proceeding, health oversight activities and public health measures.

**Right to Withdraw Your Authorization**

Your authorization for the use and disclosure of your health information for this study shall not expire unless you cancel this authorization. Your health information will be used or disclosed as long as it is needed for this study. However, you may withdraw your authorization at any time provided you notify the UNM investigators in writing. To do this, please send letter notifying them of your withdrawal to:

Christine Mermier Ph.D.
Please be aware that the research team will not be required to destroy or retrieve any of your health information that has already been used or shared before your withdrawal is received.

Refusal to Sign

If you choose not to sign this consent form and authorization for the use and disclosure of your PHI, you will not be allowed to take part in the research study.

Whom can I call with questions or complaints about this study?

If you have any questions, concerns or complaints at any time about the research study, contact the PI at 505-277-2664.

If you need to contact someone after business hours or on weekends, please call and ask for Jeremy McCormick at 505-350-8370.

If you would like to speak with someone other than the research team, you may call the UNM Office of the IRB at (505) 277-2644.

Whom can I call with questions about my rights as a research participant?

If you have questions regarding your rights as a research participant, you may call the UNM Office of the IRB (OIRB) at (505) 277-2644. The OIRB is a group of people from UNM and the community who provide independent oversight of safety and ethical issues related to research involving human participants. For more information, you may also access the OIRB website at http://research.unm.edu/irb/.

CONSENT AND AUTHORIZATION

You are making a decision whether to participate (or to have your child participate) in this study. Your signature below indicates that you/your child read the information provided (or the information was read to you/your child). By signing this consent form, you are not waiving any of your (your child’s) legal rights as a research participant.

I have had an opportunity to ask questions and all questions have been answered to my satisfaction. By signing this consent form, I agree to participate (or let my child participate) in this study. A copy of this consent form will be provided to you.

_________________________________________________
Name of Adult Subject (print)
INVESTIGATOR SIGNATURE

I have explained the research to the participant and answered all of his/her questions. I believe that he/she understands the information described in this consent form and freely consents to participate.

_________________________________________________  ____________________
Signature of Investigator/ Study Team Member Date

________________________________________________________________________
Name of Investigator/ Study Team Member (print)
APPENDIX B

Male and Female subjects needed for a research project at the UNM Exercise Physiology Lab!
HRPO# 14-200

What is the study about? This research is studying the effects of a moderate intensity aerobic exercise session on response markers of autophagy in those with and without glucose intolerance. Autophagy is a beneficial cell survival process that is induced during exercise.

Who can volunteer? Males and females between the ages of 18-55 yrs who have a normal or impaired fasting blood glucose (as indicated by a recent Hemoglobin A1C test).

What will I be asked to do if I participate? HbA1c (if not recent), body fat percentage, VO₂ max test, and submaximal exercise. Total time required for the study is 8 hours over a period of 2-3 days.

Is there any compensation for completing this study? Yes, all subjects who complete the study will receive $25.00 per exercise session in the form of a gift card (Total: $50).

Who can I contact for more information? Jeremy McCormick, 505-350-8370, aeneid@unm.edu; Christine Mermier, cmermier@unm.edu
APPENDIX C

Get $50 to Exercise!

A new study at UNM is seeking interested males and females between the ages of 18-55 who have impaired fasting blood glucose (as indicated by a hemoglobin A1C test done within the past 6 months with a value of 6.5 or above). This research is studying the effects of a moderate intensity aerobic exercise session on response markers of autophagy in those with glucose intolerance. Autophagy is a beneficial cell survival process that is induced during exercise. The study requires a total of 8 hours over a period of 2-3 days at the exercise physiology lab in Johnson center. Testing will consist of hemoglobin A1C (if not recent), body fat percentage, VO$_2$max test, and submaximal exercise on a bike. Additionally, subjects are paid $25 following the completion of each exercise session in the form of a gift card ($50 total). If interested, contact Jeremy McCormick at 505-350-8370 or aeneid@unm.edu or Christine Mermier, cmermier@unm.edu.
APPENDIX D

HEALTH HISTORY QUESTIONNAIRE (RESEARCH ONLY 5/20/02)

Subject #_____________________________ Date___/___/___

Phone #: home________________________ cell_______________

Date of Birth ___/___/___  Age____  Gender____  Ethnicity_______  Phone (W)_____________

Address (home)_____________________________________________________________ zip________

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  _____
Trygylcerides>150 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____________________

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.

This subject meets the medical screening criteria to participate in this study? Y  N

Name of Investigator/ Research Team Member (print)

______________________________  __________________________

(Signature of Investigator/ Research Team Member) Date

Name of Principal Investigator (print)

______________________________  __________________________

(Signature of Principal Investigator) Date
APPENDIX E

The Effect of a Single Exercise Session on Markers of Autophagy in Insulin-Resistant

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<td>Waist Circumference (cm):</td>
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<tr>
<td>Wt (kg):</td>
<td>HbA1C:</td>
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General Test Comments

Technician Initials: _____________________________
APPENDIX F

The Effect of a Single Exercise Session on Markers of Autophagy in Insulin-Resistant

| Subject Number: | Study Number: __________________________ |
| Age: | Date: __________________________ |
| Ht (cm): | 50% of VO2max: __________________________ |
| Wt (kg): | Starting Workload (Watts): |

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<tr>
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General Test Comments

Technician Initials: __________________________
Below is a figure that shows a comparison of basal autophagy levels and HSP72. This was omitted from the chapter 3 manuscript, as basal comparisons are made using absolute protein densitometric values from the Western blots rather than relative comparisons. Thus, the accuracy of this comparison is purely for speculative purposes.

Figure 1 Baseline regulation of protein markers of autophagy and heat shock response
Protein content of (a) LC3-II, (b) p62/SQSTM1, (c) LC3-II/I, and (d) HSP72 in peripheral blood mononuclear cells of patients with prediabetes (black bars) and control individuals (white bars) under baseline conditions. (e) Representative blots (C, control; P, prediabetes). Data are means ± S.E.M.; * indicates p < 0.05 compared with respective control.
APPENDIX H

Subject demographics with all included p-values. This was included to show non-statistically significant differences between groups.

Table 1 Clinical and metabolic characteristics at study entry
Data are presented as means ± SD. * P < 0.05 compared with control.

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Prediabetes</th>
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<tr>
<td>n</td>
<td>6</td>
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<tr>
<td>Age (years)</td>
<td>42.4 ± 11.7</td>
<td>44.4 ± 11.9</td>
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<td>BMI (kg/m²)</td>
<td>23.7 ± 2.5</td>
<td>24.2 ± 5.6</td>
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<td>Body Fat (%)</td>
<td>23.5 ± 7.2</td>
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<td>Waist Circumference (cm)</td>
<td>80.8 ± 3.5</td>
<td>83.9 ± 15.3</td>
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<td>VO₂max (ml/kg/min)</td>
<td>38.6 ± 9.1</td>
<td>36.8 ± 6.8</td>
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<td>HbA1c (%)</td>
<td>5.3 ± 0.1</td>
<td>6.0 ± 0.2*</td>
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</table>