

Skeletal muscle autophagy and mitophagy in response to high-fat feeding and endurance training

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ABSTRACT

Obesity is associated with reduced skeletal muscle insulin sensitivity, a major risk factor for development of type II diabetes. These metabolic diseases are commonly associated with an accumulation of mitochondrial dysfunction, which is speculated to contribute toward insulin resistance. High-fat diets reduce human skeletal muscle insulin sensitivity and mitochondrial function. Conversely, endurance training increases insulin sensitivity and enhances mitochondrial performance. Recent evidence in mice has found that central mechanisms of mitochondrial quality control, autophagy and mitophagy, may be suppressed in response to excess fat intake, but upregulated following endurance exercise training. These data may provide a mechanism for dietary and exercise-mediated regulation of mitochondrial quality and metabolic function. The current study investigated the impact of an acute high-fat diet on skeletal muscle autophagy and mitophagy in sedentary, healthy, non-obese college age males'. The expression of skeletal muscle autophagy and mitophagy protein markers were analyzed in response to a high-fat meal before and after a 5-day high-fat diet. Next, we examined the differences in skeletal muscle autophagy and mitophagy protein markers, and associations with skeletal muscle metabolic flexibility between endurance-trained male runners' and sedentary, healthy, non-obese males' following an overnight fast and in response to a high-fat meal. Autophagy markers' indicated reduced autophagy activity in response to a high-fat meal and following a high-fat diet, which exacerbated the high-fat meal response. However, these data could not be confirmed due to methodological limitations. Mitophagy markers were not significantly affected by the high-fat meal or diet. There were no significant differences in the expression of autophagy protein markers between endurance-trained

and sedentary groups', but mitophagy markers were significantly elevated in endurance-trained runners'. Metabolic flexibility was not significantly different between groups' following an overnight fast or in response to a high-fat meal, and was not associated with the expression of autophagy and mitophagy protein markers. In conclusion, autophagy may be suppressed by a 5-day high-fat diet, but further analysis is required for confirmation. Endurance-trained male runners show increased markers of mitophagy, which were not associated with improved metabolic flexibility while fasted or following a high-fat meal.

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ATTRIBUTION

Several colleagues aided in the design of studies, collection of data and preparation of manuscripts for two chapters presented as part of this dissertation. A brief description of their contributions is given here.

Chapter IV: The effect of a 5-day high-fat diet on skeletal muscle autophagy and mitophagy

Kris Osterberg, Ph.D., was responsible for screening participants' and diet development. Suzanne Bowser and Ryan McMillan, Ph.D., aided in the collection of data for this chapter. Kevin Davy, Ph.D. aided in data collection and manuscript preparation. Matthew Hulver, Ph.D., was responsible for study design and aided in data collection and manuscript preparation. Madlyn Frisard, Ph.D., aided in preparation of the manuscript.

Chapter V: Skeletal muscle autophagy and mitophagy in endurance-trained runners, and in response to a high-fat meal

Suzanne Bowser and Ryan McMillan, Ph.D., aided in the collection of data for this chapter. Tanya Halliday provided analysis of dietary intake. Matthew Hulver, Ph.D., and Kevin Davy, Ph.D., aided in the study design, data collection and preparation of the manuscript. Madlyn Frisard, Ph.D., aided in participant recruitment, study design and manuscript preparation.

CHAPTER I

INTRODUCTION

World-wide, the prevalence of obesity and type II diabetes mellitus (T2D) has increased dramatically over the past three decades and is now a well-recognized epidemic with 2.1 billion overweight and obese adults (Ng et al., 2014). Moreover, if recent secular trends continue uninterrupted then there may be approximately 3.3 billion overweight and obese adults globally by 2030 (Kelly et al., 2008). A key feature of obesity is insulin resistance (Qatanani and Lazar, 2007; Wahba and Mak, 2007), which appears 10-20 years prior to the onset of T2D and is therefore a major predictor of disease development (Lillioja et al., 1988; Warram et al., 1990). The association between obesity, insulin resistance, and T2D is therefore well established, in fact, excess body weight is a factor in 90% of patients with T2D (Guh et al., 2009). The health of these individuals is further compromised by a greater risk of developing associated co-morbidities including hypertension and cardiovascular disease, leading to an increased mortality (Zimmet, Alberti and Shaw, 2001; Sullivan et al., 2005). Recent estimates suggest there are already 387 million diagnosed and undiagnosed T2 diabetic adults world-wide (IDF Diabetes Atlas, 6th edn, 2013), which combined with the growth in overweight and obese individuals represents a burgeoning global health crisis.

Obesity and T2D also represent a significant financial burden. In the U.S., direct medical costs of obesity are estimated to account for 5-10% of total national healthcare expenditure (Tsai et al., 2011), and predicated to rise to between 16% and 18% by 2030 (Wang et al., 2008). Meanwhile, the total economic cost of diagnosed diabetes to the U.S., of which T2D represents 95% of cases (CDC: National Diabetes Statistics Report, 2014), was estimated to be \$245 billion in 2012

(American Diabetes Association, 2013). Future costs may increase substantially as recent trends predict the prevalence of T2D will be 1 in 3 by 2050 (Boyle et al., 2010). Considering the current and impending human and financial costs associated with obesity and T2D it is vital that investigations focus on further understanding the underlying mechanisms and early stages of these diseases to enhance existing prevention and treatment strategies, as well as developing novel approaches.

Mitochondria are versatile organelles, central to energy metabolism through the production of ATP, generation and elimination of reactive oxygen species (ROS), and as regulators of basal substrate selection (Gottlieb and Carreira, 2010; van de Weijer et al., 2013). Obese, insulin resistant and T2D patients show impaired skeletal muscle mitochondrial function, leading to reductions in oxidative capacity and increased ROS production (Kelley et al., 2002; Mootha et al., 2003; Ritov et al., 2010; Wijngaarden et al., 2013). Furthermore, obesity and insulin resistance-inducing high-fat, high-saturated fat diets (HF/HSFD) are a cause of mitochondrial dysfunction in skeletal muscle of rodents (Bonnard et al., 2008; van der Broek et al., 2009; Jheng et al., 2012; Yuzefovych et al., 2013). Reduced skeletal muscle mitochondrial function may therefore be a factor in the development and progression of metabolic disease.

Maintenance of mitochondrial quality, and thus function, is dependent on the elimination of damaged, dysfunctional mitochondria and synthesis of new, healthy mitochondria, through the coordinated interactions of mitochondrial biogenesis and mitophagy (Gottlieb and Carreira, 2010; Patel, Shirihai and Huang, 2013). Mitophagy describes the selective degradation of mitochondria through autophagy (Lemasters, 2005). Mitochondria derived from skeletal-muscle specific

autophagy knockout mice exhibit morphological and functional impairments (Wu et al., 2009; Moresi et al., 2012; Kim et al., 2013) that may indicate reduced mitophagy, and thus a loss of mitochondrial quality control. Skeletal muscle autophagy is also suppressed followed a HF/HSFD in mice, which developed obesity and impaired glucose homeostasis (He et al., 2012). HF/HSFD-induced autophagy suppression, leading to reductions in mitophagy and the eventual loss of mitochondrial quality control may therefore be a factor in the etiology of obesity, insulin resistance and T2D. However, the effects of HF/HSFD on human skeletal muscle autophagy and mitophagy are not understood.

In addition to diet, a sedentary lifestyle is a prominent factor in the etiology of obesity, insulin resistance and T2D (Swinburn et al., 2004; Kaur, 2014). Conversely, exercise training is associated with reduced body mass index (BMI) (Marandi et al., 2013) and increased insulin sensitivity in healthy adults (Dela et al., 1992; Houmard et al., 1993; Evans et al., 2001), as well as improved insulin sensitivity in obese (Goodpaster, Katsiatas and Kelley, 2003; Shojaee-Moradie et al., 2007) and T2 diabetic patients (Dela et al., 1995). Exercise is also a modulator of autophagy in rodent (Grumati et al., 2011; Jamart et al., 2013; Lira et al., 2013) and human skeletal muscle (Jamart et al., 2012a and 2012b; Moller et al., 2015). Chronic exercise training leads to increased basal autophagy and mitophagy in murine skeletal muscle (Lira et al., 2013), but it is unknown if human skeletal muscle responds similarly. The consequences of enhanced basal autophagy and mitophagy for metabolic disease prevention and progression are not clear, but may support a healthier mitochondrial population that ultimately increases skeletal muscle capacity to withstand metabolic insults such as an HF/HSFD. Further research is required to disseminate the impact of HF/HSFD on human skeletal muscle autophagy and mitophagy, and whether changes are associated with

impaired metabolic regulation. Investigations into the effects of exercise training on autophagy and mitophagy in human skeletal muscle are also required to further understand if and how these processes may prevent and repair defects to metabolic regulatory pathways. This dissertation will aim to explore and address these literary gaps through a systematic review of the literature and preliminary investigations into the interaction between HF/HSFD and human skeletal muscle autophagy and mitophagy.

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CHAPTER II

Systematic Review of Literature

Scope of Review

The review will aim to provide an overview of autophagy; its regulatory mechanisms and involvement in metabolic regulation, particularly within skeletal muscle. Next, attention will be paid to investigating the regulation of mitochondrial dynamics and mitophagy with particular attention to how these systems support mitochondrial quality control. The review will explore how mitophagy and mitochondrial dynamics are integrated with bioenergetics, and the current understanding of how these processes relate to diet and exercise in the context of skeletal muscle based cell culture and transgenic models. Finally, the literature will be systematically searched for studies investigating skeletal muscle mitophagy and mitochondrial dynamics in response to diet and exercise. The findings will be reviewed to highlight current knowledge and gaps in the literature.

Summary of findings

The literature is thin, but skeletal muscle mitophagy and mitochondrial dynamics seem sensitive to HFD consumption, which may alter mitochondrial quality control. Loss of mitochondrial function is not unanimously associated with HFD-induced insulin resistance, but further investigation is warranted. Importantly, mitochondrial bioenergetics related signaling may sensitize mitophagy and mitochondrial dynamics to shifts in skeletal muscle oxidative capacity. Mitophagy responds to acute and chronic exercise in a fiber-type-dependent manner and may mediate the adaptive response.

Conclusion

The area of skeletal muscle mitophagy and mitochondria dynamics has not been extensively studied and represents a literary niche. This is particularly true for how mitophagy and dynamics maintain skeletal muscle mitochondrial quality control in response to high-fat diets and exercise. Further preliminary research is required to better understand how mitophagy and mitochondrial dynamics are involved in the skeletal muscle response to high-fat diet and exercise. This will provide the necessary knowledge to develop more elaborate studies to examine implication for metabolic disease.

Autophagy

Autophagy in the general sense encompasses microautophagy, chaperone-mediated autophagy and macroautophagy (to be referred to as autophagy), the focus of the current review. Briefly, microautophagy describes the degradation of cell constituents isolated inside single-membrane vesicles within the lysosome (Codogno and Meijer et al., 2005; Yamada and Singh, 2012), while chaperone-mediate autophagy selectively targets soluble cytosolic proteins for degradation (Codogno and Meijer et al., 2005; Yamada and Singh, 2012). The autophagic route of protein degradation is lysosome-dependent. A second, non-lysosome-dependent, mechanism of protein degradation, the ubiquitin-proteasome system, is also active in cells. In short, the ubiquitin-proteasome system tags protein targets with adenosine triphosphate activated ubiquitin, catalyzed by enzymes E1, E2 and E3, before proteolysis via the 26S proteasome (Bhattacharyya et al., 2014). The current review will focus on autophagic degradation, discussing its regulation and mechanism

of action due to its role in mitochondrial quality control. Evolutionarily conserved, autophagy coordinates cellular quality control processes that rely on lysosome digestion for the turnover of cytosolic constituents (Codogno and Meijer et al., 2005; Yamada and Singh, 2012). Autophagy can be subdivided into target-dependent classifications including ER-phagy (endoplasmic reticulum degradation), pexophagy (peroxisome degradation), xenophagy (pathogen degradation) and, of particular interest, mitophagy (mitochondrial degradation).

Overlap in the molecular machinery between autophagy, apoptosis and even necrosis may give the impression that autophagy is predominantly associated with cell death. However, evidence suggests that autophagy is also prominent in cell health and survival (Rikka et al., 2011; Kubli and Gustafsson, 2012). Autophagy can promote cell survival through the provision of amino acids (AA) during periods of fasting and starvation by turning over non-essential proteins and organelles (Lee et al., 2014). Secondly, autophagy provides a mechanism of removing dysfunctional organelles such as mitochondria to prevent cell damage and apoptosis (Rikka et al., 2011). Autophagy can operate under two states, a non-stimulated or basal state, and a stimulated state (Musiwaro et al., 2013). Basal autophagy is constitutively active at low rates even under nutrient-rich conditions and is required for the fundamental turnover of cytosolic components such as organelles (Komatsu et al., 2007; Mizushima, 2007). Periods of fasting and nutrient starvation are commonly studied stimulated states during which autophagy operates as a survival mechanism by producing AAs (Komatsu et al., 2007; Musiwaro et al., 2013).

As discussed above, autophagy can operate in a basal or stimulated state. While there is considerable overlap in the pathways and proteins utilized by both forms to sequester and degrade

cytoplasmic constituents, the processes of activation and regulation differ. The most studied form of autophagy is that of the stimulated state, using models of nutrient deprivation. Comparatively little is understood about the regulation of basal autophagy, although the main difference between basal and stimulated autophagy appears to be the initial activation step. Under basal conditions anti-autophagy Bcl-2 is bound to pro-autophagy Beclin-1, rendering it dormant (Pattingre et al., 2005). Basal autophagy does not therefore require Beclin-1 to be active, whereas the dissociation of Bcl-2 and Beclin-1 is critical for stimulated autophagy (Liang et al., 1998; He and Levine, 2010). A recent paper (Oh et al., 2015) suggested that in human embryonic kidney cells (HEK293 cells) basal autophagy may instead be modulated by Ca^{2+} influx via the TRPM7 Ca^{2+} transporter. The authors reported that TRPM7-mediated Ca^{2+} influx activated basal autophagy via the CaMKK β / AMPK pathway in a nutrient-rich environment. However, the findings were based in part on manipulating either TRPM7 or Ca^{2+} concentrations, leading to increases in Ca^{2+} influx that positively correlated with changes in autophagy activity. It has previously been reported that Ca^{2+} influx occurs during starvation and stimulates autophagy through the CaMKK β / AMPK pathway (Ghislat et al., 2012). Oh et al., (2015) did not control for stimulated-autophagy pathways, which were left intact. It is possible therefore that a portion of the data reported represented stimulated autophagy due to artificial increases in cellular Ca^{2+} concentrations, and not basal autophagy. Although, reductions in passive Ca^{2+} influx through TRPM7 demonstrated a reduction in autophagy under nutrient-rich conditions, which would suggest some involvement with basal autophagy assuming the cells were not already subject to stimulated autophagy through some stress other than starvation. The regulation of basal autophagy through Ca^{2+} is further complicated by findings that chronically elevated cytosolic Ca^{2+} concentrations in mouse liver cells reduce autophagy by interfering with autophagosome and lysosome fusion (Park et al., 2014).

Pharmacological inhibition of Ca²⁺ channels led to a reduction in cytosolic Ca²⁺ concentrations and a restoration of autophagy (Park et al., 2014). The role of Ca²⁺ in basal autophagy regulation therefore remains unclear.

Stimulated autophagy, the more studied of the two forms, presents a clearer picture. The initiation of stimulated autophagy proceeds via disruption of the Bcl-2/ Beclin-1 complex. The factor responsible depends up on the specific form of stress leading to the stimulation of autophagy. Under starvation-induced stress Bcl-2 is phosphorylated at Thr69, Ser70 and Ser87 by c-Jun N-terminal kinase 1 (JNK1) causing it to dissociate from Beclin-1, and autophagy to progress (Wei et al., 2008). The process of dissociation is further driven through phosphorylation of Beclin-1 at Ser14 by ULK1 (unc-51 like autophagy activating kinase 1) (Russell et al., 2013). The activation of ULK1 is due to reduced phosphorylation of its inhibitory Ser757 residue, stemming from suppressed mTORC1 activity (Vendelbo et al., 2014). Interestingly, exercise-induced autophagy requires phosphorylation of the same residues on Bcl-2, as that of starvation-induced autophagy, but not via JNK1 (He et al., 2012). Recently, it was discovered that exercise-induced autophagy in human skeletal muscle is partially mediated through phosphorylation of ULK1 Ser555, an exercise-specific phosphorylation site that activates ULK1 (Moller et al., 2015). The target of activated ULK1 in response to exercise is however not clear. Data from starvation-induced autophagy (Russell et al., 2013) might suggest that ULK1 mediates exercise-induced autophagy through Beclin-1 phosphorylation. This alone though seems implausible since transgenic Bcl-2 AAA mice, that have alanine residues in place of Thr69, Ser70 and Ser87, are unable to activate autophagy in response to exercise (He et al., 2012). This could indicate ULK1 phosphorylates Bcl-

2 in response to exercise, in place of Beclin-1, or that phosphorylation of Bcl-2 and Beclin-1 is required for autophagy.

Reactive oxygen species (ROS) may also rely of Bcl-2/ Beclin-1 dissociation to initiate autophagy, as evidence suggests that ROS production occurs upstream of Beclin-1 activation (Chen, Azad and Gibson, 2009) and converts the catalytic cysteine residues of JNK-inactivating phosphatases to sulfenic acid, causing sustained JNK activation (Kamata et al., 2005). In addition, ROS may further disrupt Bcl-2/ Beclin-1 binding through the transcription of BNIP3 (Bcl-2/adenovirus E1B-19kDa interacting protein 3) and NIX/ BNIP3L (BCL2/adenovirus E1B 19kDa interacting *protein* 3-like), which competitively bind Bcl-2 in place of Beclin-1 (Zhang et al., 2008; Bellot et al., 2009). Increased transcription of BNIP3 and NIX is the result of ROS-induced stabilization of hypoxia-inducible factor-1 (HIF1) (Chandel et al., 2000; Guzy et al., 2005). Although the stabilizing effect appears to be biphasic, and as such ROS acts as a short term autophagy stimulation that becomes suppressive with prolonged exposure (Niecknig et al., 2012). Additional disruptors of the Bcl-2-Beclin-1 complex include HMGB1 (high mobility group protein 1), DAPK (death-associated protein kinase) and Ambra-1 (activating molecule in BECN1-regulated autophagy protein 1). HMGB1 interacts with both Bcl-2 and Beclin-1 to promote autophagy (Decuypere, Parys and Bultynck, 2012). Oxidation of specific cysteine residues on HMGB1 under mild oxidative stress, promote the formation of disulfide bridges, which are required for the binding of HMGB1 to Beclin-1, and sustained autophagy (Hoppe et al., 2006; Tang et al., 2010). The role of DAPK in autophagy has not been clearly defined, although its mechanism of action seems mediated through phosphorylation of Beclin-1 at Thr119, which resides within the BH3 binding domain required for association with Bcl-2, thus triggering the release of Beclin-1 (Zalckvar et al., 2009). For now,

DAPK is known to be associated with starvation-induced autophagy (Kang, You and Avery, 2007). Ambra-1 is a positive regulator of autophagy during periods of starvation-induced stress through its interaction with Beclin-1 (Kang et al., 2011; Strappazzon et al., 2011). This interaction is regulated through binding of Ambra-1 with Bcl-2, thus preventing translocation of Ambra-1 to Beclin-1 (Strappazzon et al., 2011). The inhibitory effect of Bcl-2 on Ambra-1 does not occur through endoplasmic reticulum-bound Bcl-2, but is instead specific to mitochondrial localized Bcl-2 (Strappazzon et al., 2011). Conversely, the inhibitory effect of Bcl-2 on Beclin-1 is limited to ER localized Bcl-2 (He and Levine, 2010), while Ambra-1 is capable of interacting with Beclin-1 at both ER and mitochondria (Strappazzon et al., 2011), the latter of which has been suggested as a source of autophagosomal membrane. Moreover, the localization of Beclin-1 to the omegasome (site of autophagosome formation) on the ER may be directed by phosphorylated Ambra-1, under the control of ULK1 (Nazio and Cecconi, 2013). The consequence of this location-specific regulation of autophagy is not clear, but may represent a control point between autophagy and apoptosis since Bcl-2 and Ambra-1 binding is reduced during apoptosis (Strappazzon et al., 2011) and mitochondria are key to apoptosis activation (Wang and Youle, 2009).

Following the dissociation of Beclin-1 from Bcl-2, formation of the nucleation complex is initiated through the transient binding of Beclin-1 to vacuolar protein sorting (vps) 15 and the lipid kinase vps34, which phosphorylates phosphatidylinositol producing phosphatidylinositol 3-phosphate (PtdIns3P) (Codogno and Meijer et al., 2005; Decuypere, Parys and Bultynck, 2012; Yamada and Singh, 2012). This generates a pro-autophagic functional class III protein complex, which facilitates the subsequent recruitment of Atg (autophagy gene) molecules to the nucleation complex (Codogno and Meijer et al., 2005; Yamada and Singh, 2012). The second phase, aided

by the transmembrane protein Atg9, forms the limiting membrane and promotes its elongation (Codogno and Meijer et al., 2005; Yamada and Singh, 2012). Atg7, an E1-like activating enzyme is responsible for the tandem catalyzing of Atg5-Atg12 binding and activation of the microtubule-associated protein 1 light chain 3 (LC3) to its cytosolic LC3I form (Codogno and Meijer et al., 2005; Yamada and Singh, 2012). Interactions between the Atg5-Atg12 complex and Atg16 drafts these proteins to the limiting membrane (Codogno and Meijer et al., 2005; Yamada and Singh, 2012). Meanwhile, LC3I is transferred to Atg3 and converted to its limiting membrane bound phosphatidylethanolamine (PE) conjugated form, LC3II, through a series of Atg5-Atg12-dependent reactions (Codogno and Meijer et al., 2005; Yamada and Singh, 2012). The limiting membrane ultimately closes on itself, trapping autophagy substrates within the double membrane autophagosome. Lysosome fusion acidifies the lumen before a series of lysosome permeases and transporters release the hydrolysed protein remnants into the cytosol (Codogno and Meijer et al., 2005; Yamada and Singh, 2012).

Autophagy and metabolism are intricately entwined, allowing an adaptive autophagic response to changes in nutrient availability and exercise. The regulatory mechanisms governing this relationship are not completely known, but appear to revolve around an insulin-Akt-mTORC1-ULK1 pathway (Jamart et al., 2013). Insulin action through insulin receptor substrate (IRS) activates Akt and subsequently phosphorylates mTORC1^{Ser2448} (mammalian target of rapamycin complex 1) (Siddle, 2011). The central regulator of autophagy, mTORC1, integrates environmental cues to operate as a master switch for the stimulation and suppression of anabolic and catabolic cell pathways, respectively (Magnuson, Ekim and Fingar, 2012). Phosphorylation of mTORC1 suspends the complex in its active form, functioning as a brake on the autophagic

pathway through covalent modification of downstream targets (Ekim et al., 2011). Of significance, ULK1 the human homologue of Atg1 is phosphorylated at Ser757 by active mTORC1, rendering it dormant within the autophagosome biogenesis-dependent Atg13-FIP200-ULK1^(Ser757) complex (Yamada and Singh, 2012). Removal of mTORC1 inhibition, such as occurs during starvation, promotes activity of the ULK1 (Hosokawa et al., 2009). In addition to ULK1's role in Bcl-2 and Beclin-1 dissociation, ULK1 is also capable of binding LC3 for autophagosome-targeted delivery through the cooperative action of Atg13 (Kraft et al., 2012), allowing the shuttling of Atg9. ULK1 is not exclusively regulated through mTORC1, but also via adenosine monophosphate- activated protein kinase (AMPK), the master regulator of cellular energy homeostasis (Dunlop and Tee, 2013) and activating kinase for JNK1-mediated disruption of the Bcl-2/ Beclin-1 complex (He et al., 2013). In the case of ULK1, energy depletion and starvation-induced activation of AMPK results in phosphorylation of AMPK-dependent ULK1 phosphorylation sites (Ser555 and Ser317), promoting autophagy (Ghislat et al., 2012; Jamart et al., 2013). Additionally, AMPK can act on ULK1 through mTORC1 inhibition (Dunlop and Tee, 2013). mTORC1 is sensitive to cellular AA concentrations, adjusting autophagic flux accordingly, while AMPK, also sensitive to nutrient status, is a prominent exercise-induced kinase. The ability of both master switches to regulate ULK1 may provide the cell a means of adapting autophagy in response to changes in energy availability and demand. Dunlop and Tee (2013) suggest a similar mode of regulation, although with ULK1 negatively feeding back on AMPK through phosphorylation of all three AMPK subunits (Loffler et al., 2011), forming the mTORC1-AMPK-ULK1 kinase triad. This appears to provide a compensatory mechanism to safe guard against constitutively active autophagy (Dunlop and Tee, 2013). Jamart et al., (2013) found that autophagy activation was more prominent in mice when exercising in a fasting state, compared to a fasting state alone. However, AMPK^{Thr172}

phosphorylation was unaltered between fasting and fasting state exercise in mice, as were ULK1^{Ser317} and ULK1^{Ser555}. The mTORC1 regulated inhibitory Ser757 phosphorylation site of ULK1 on the other hand was significantly reduced when exercise in a fasting state compared to fasting alone, potentially indicating a more prominent role for mTORC1 in ULK1 and autophagy regulation.

Further mTORC1 downstream targets include S6K (ribosomal protein S6 kinase) and 4E-BP1 (eukaryotic translation initiation factor 4E binding protein). The opposing pathway to that of autophagy is protein synthesis and through S6K and 4E-BP1, mTORC1 regulates cell growth and proliferation (Magnuson, Ekim and Fingar, 2012). These targets have previously been used as markers of mTORC1 activity in studies analyzing dietary and exercise effects on autophagy and mitophagy (Jamart et al., 2013; Jamart et al., 2012). In light of the current review's scope, a detailed description of S6K and 4E-BP1 signaling is not necessary. In short, autophagy is suppressed under conditions of S6K phosphorylation, when mTORC1 is active (Ekim et al., 2011; Magnuson, Ekim and Fingar, 2012). S6K is phosphorylated under an anabolic state, such as that following a meal when insulin levels are high and autophagy is suppressed. It has been suggested that S6K may in fact indirectly activate autophagy by negative feedback on insulin/ PI3K signaling (Magnuson, Ekim and Fingar, 2012). The insulin signaling mediator Akt (known also as protein kinase B) is phosphorylated by phosphatidylinositol 3-kinase (PI3K) in response to insulin activation of IRS1 (insulin receptor substrate) (Hemmings and Restuccia, 2012), leading to its activation and inhibition of autophagy through phosphorylation of mTORC1^{Ser2448} (Nave et al., 1999). Therefore, through negative feedback on insulin/ PI3K signaling, S6K may alleviate insulin-induced suppression of autophagy (Codogno and Meijer, 2005; Yamada and Singh, 2012).

This may be a mechanism by which the body regulates and sustains basal autophagy even under anabolic conditions. This would also indicate that under insulin resistant and diabetic conditions, autophagy should increase as a result of reduced insulin/ PI3K signaling and resultant reduction in Akt-mediated phosphorylation of mTORC1^{Ser2448}. In fact, Codogno and Meijer (2005) proposed that autophagy may be elevated in type II diabetic patients due to the loss of insulin's suppressive effect and that this may function as a protective mechanism in older patients.

Thus far the review has discussed the stepwise progression of autophagy and posttranscriptional regulation of autophagy through its numerous modulators. A further consideration is that of transcription factors. Forkhead box O3a (FoxO3a) is a transcription factor responsible for the upregulation of key autophagy related proteins in skeletal muscle, including LC3 and BNIP3, the latter protein now also commonly associated with mitophagy (Mammucari et al., 2007). FoxO3a is predominantly under the control of Akt, which covalently blocks its translocation to the nucleus, reducing autophagy gene transcription (Yamada and Singh, 2012; Jamart et al., 2013). Given the central role of mTORC1 in autophagic regulation it too has been proposed as a possible controller of FoxO3a, however, in vivo administration of rapamycin did not influence Akt-induced suppression of FoxO3a (Mammucari et al., 2007). These data suggest FoxO3a is independent of mTORC1, or FoxO3a is regulated via the rictor subunit containing mTORC2, which is rapamycin insensitive (Codogno and Meijer, 2005). Further investigation revealed that mTORC2 does appear to have an inhibitory influence over FoxO3a (Mammucari et al., 2007), possibly through an Akt-dependent feedback loop. Mentioned above was the impact that insulin resistance and/ or diabetes may have on autophagic activity. Given that hypothesized increases in autophagy under insulin resistance states is dependent upon reduced Akt activity, and thus loss of posttranscriptional

autophagy suppression, it seems likely that autophagy would be increased transcriptionally under insulin resistant conditions. While there is a mechanistic possibility that insulin resistance may lead to altered autophagic regulation and activity, it is not clear whether such alterations would be detrimental or protective, or whether dysregulated autophagy could precede insulin resistance and diabetes.

Autophagy and metabolism in non-skeletal muscle tissues

In response to fasting and short term starvation, upregulation of autophagy provides the cell with essential nutrients for ATP production and anabolic pathways through the digestion of nonessential constituents (Kuma et al., 2004; Mizushima et al., 2004; Komatsu et al., 2007). Interestingly, animal models have demonstrated that calorie restriction increases life span (Weindruch and Sohal, 1997; Liao et al., 2010), possibly through reduced reactive oxygen species production (Leeuwenburgh et al., 1997). In circumstances of heightened oxidative stress mitochondria are susceptible to deleterious alterations such as loss of membrane potential or mtDNA mutations (Wohlgemuth et al., 2010). These mitochondria represent a metabolically compromised population, the removal of which is essential to avoiding the release of mitochondrial bound cell death-inducing factors (Rikka et al., 2011). It may be that greater autophagy under calorie restricted conditions increases the turnover rate of damaged mitochondria, thus reducing oxidative stress. Alternatively, calorie restriction may directly reduce ROS production thus reducing cellular damage and aging, with the greater autophagic flux simply increasing protein turnover to compensate for reduced consumption.

A further sub-category of autophagy is lipophagy, a process describing the autophagosome delivery of lipids for lysosomal degradation (Lee et al., 2014). Little is understood about the process in skeletal muscle, however, studies have been conducted in liver and adipose tissue. Studies conducted in liver-specific Atg7 KO mice demonstrated an accumulation of lipids in liver, which was found not to be the result of altered biogenesis (Yang et al., 2010). As might be expected, lipophagy was greater under conditions of starvation, supporting its role in the production of free fatty acids for beta-oxidation (Yang et al., 2010). A further finding of interest was that while acute lipid loading in cells activated lipophagy, chronic high-fat feeding blunted hepatic lipophagy (Yang et al., 2010), which suggests high-fat diets (HFDs) might lead to autophagy deficiency. Indeed, Yang et al., (2010) subsequently showed that HFD-induced obese mice exhibited hepatic autophagy deficiency following 22 weeks of HFD, leading to endoplasmic reticulum-induced insulin resistance. This raises the question of whether HFDs could have a similar effect in other tissues such as skeletal muscle. Several theories have been proposed to explain the phenomenon. Firstly, the accumulation of intracellular lipids and fatty acid intermediates, commonly reported in obese and diabetic patients, may alter membrane lipid composition leading to dysfunctional autophagosome-lysosome fusion (Koga et al., 2010). Additionally, hyperactivation of the autophagy suppressor, mammalian target of rapamycin (mTOR) may be responsible, an effect characteristic of obesity (Schloesser et al., 2014). It remains to be conclusively determined whether a similar event occurs in skeletal muscle as a result of high-fat feeding and if depressed autophagy contributes to insulin resistance, particularly in humans.

In contrast to the effects of autophagy deficiency in liver, which appears to produce primarily negative metabolic modifications, including lipid accumulation and insulin resistance, adipose

tissue responds quite differently. Adipose-tissue specific Atg7 KO mice are characterized by reduced body weight and total fat mass, and increased insulin sensitivity and beta-oxidation (Singh et al., 2009; Zhang et al., 2009). The mice had significantly reduced white adipose tissue (WAT), but significantly greater brown adipose tissue (BAT) (Singh et al., 2009; Zhang et al., 2009). Interestingly, the WAT of the KO mice exhibited BAT characteristics, including reduced adipocyte size and substantial increases in mitochondrial content of up to 8-fold (Singh et al., 2009; Zhang et al., 2009). Mitochondrial content was also increased in BAT, although to a lesser degree. Finally, adipose-tissue specific Atg7 KO mice were more active, consumed equal calories and were resistant to HFD-induced obesity (Singh et al., 2009; Zhang et al., 2009). It is worth noting that despite these apparently positive metabolic outcomes, KO mice were infertile and had greater mortality rates (Singh et al., 2009). The current review is specifically focused on mitophagy and mitochondrial turnover in skeletal muscle, a topic detailed later in the review. It is unfortunate therefore that the studies discussed did not measure markers of mitophagy and function (described later) to determine if reduced mitophagy caused the increased mitochondrial populations in WAT and BAT with compromised function. Singh et al., (2009) did find greater PGC1 α levels in both WAT and BAT, suggesting increased mitochondrial biogenesis was at least partly responsible. The validity of the KO model was confirmed by an absence of steady-state autophagosome formation indicated by undetectable LC3II levels in the presence of high LC3I levels, a lack of functional autophagic degradation shown by the accumulation of p62, and the inability to form autophagosomes as determined by the absence of atg5-atg12 conjugate (Singh et al., 2009; Zhang et al., 2009).

Knockout Atg7 mutant mice, a popular model for tissue-specific autophagy analysis, have also been employed to investigate pancreatic autophagy. Mice with a β cell-specific Atg7 deletion exhibited reduced pancreatic insulin content and β cell mass resulting from greater apoptosis and reduced β cell proliferation (Jung et al., 2008). Impaired whole body glucose tolerance and reduced serum insulin ensued. Defective autophagy was confirmed by an accumulation of p62-colocalized ubiquitinated protein aggregates. Autophagy is therefore necessary for sustaining β cell function and mass, presumably in part through protection and rescue from apoptosis. Additional morphological decline included distention of the endoplasmic reticulum and mitochondrial swelling. Similar results were reported by Ebato and colleagues' et al., (2008) using Atg7 under-expression mice. While the tissue-specific Atg7 KO models are novel, providing important insights regarding the necessity of autophagy in homeostatic maintenance, they do not necessarily reflect environmental or biological conditions responsible for metabolic disease development. The reductionist nature of such models, although serving to isolate tissue-specific alterations may not be appropriate for understanding development and progression of metabolic diseases like obesity and diabetes, which exert systemic effects and can be the result of simultaneous dysfunction in multiple tissues and organs. Findings on the effects of HFDs, associated with the development of metabolic disease, may be of greater relevance.

Ebato et al., (2008) fed C57BL/6 mice a diet containing 60% fat for 12-weeks, demonstrating increased formation of mitochondrion and insulin granule-containing autophagosomes in β cells relative to standard chow fed mice. This finding correlated with HFD-induced obesity and insulin resistance. In light of the deleterious effects of autophagy suppression, these data may suggest that autophagy is defensively upregulated in response to obesity and insulin resistance, protecting

against more severe β cell deterioration. However, without complimentary markers of autophagic flux it is difficult to make definitive conclusions. In vitro INS-1 β cell exposure to oleate for 12 and 24 hours is associated with increased lipidation of LC3I, indicative of greater autophagic flux, an effect not witnessed with glucose (Ebato et al., 2008). In contrast, similar FFA exposure in cultured human islets shows a more significant autophagic vacuole density, but absent of any change in autophagic flux (Lupi et al., 2002).

The studies of Yang et al., (2010) and Ebato et al., (2008) demonstrate that autophagy activity may become dysregulated following a HFD or FFA exposure, but a mechanism is not clearly defined. Recently, Park et al., (2014) identified inhibited fusion between autophagosome and lysosomes as the culprit of defective autophagy in hepatocytes exposed to saturated fatty acids (SFAs). Similar results were not seen with unsaturated fatty acid (UFA) exposure, which may represent a source of controversy in HFD models. It is tempting to attribute SFA-specific autophagy dysregulation to oxidative stress because palmitate has previously been found to promote reactive oxygen species production in L6 cells, an effect blunted in the presence of UFAs (Yuzefovych et al., 2012 and 2010). However, Park et al., (2014) found the addition of ROS quenchers did not prevent defective autophagy, instead suggesting that increased cytosolic calcium was a mediating influence. Treatment of HFD-induced obese mice with the calcium channel blocker, Verapamil, restored hepatic autophagy flux and suppressed obesity-induced insulin resistance (Park et al., 2014). Given the wide range of signaling actions performed by calcium it is difficult to determine whether protection against obesity-induced insulin resistance was a direct effect of autophagy flux restoration. Additionally, Koga and colleagues (2010) found defective autophagosome and lysosome fusion in livers of 60% HFD fed mice, consistent with Park et al., (2014). However,

cultured hepatocyte experiments did not show that deficient fusion was limited to SFA. Uniquely, the study highlighted adaptations to autophagosome and lysosome lipid membranes in response to HFDs, as a mediator of autophagy dysfunction. Cholesterol content of lysosomal lumens and membranes, isolated from HFD fed mice, were significantly reduced. Under fasting state conditions, isolated fractions from HFD mice exhibited reduced fusion, which was not reproducible under post-prandial conditions. A series of cross assays revealed that fusion was inhibited when HFD autophagosomes were introduced to lysosomes isolated from control mice. Altered autophagosome membranes are therefore sufficient to reduce fusion. Curiously, a reverse combination of control autophagosomes and HFD lysosomes lead to a greater compromise of fusion compared to mixed HFD autophagosomes and lysosomes, suggestive of a compensatory mechanism (Koga et al., 2010). Further analysis of cellular lipid content, and autophagosome and lysosome membrane lipid composition, in response to HFDs may lead to improved understanding of regulatory mechanisms.

Skeletal muscle autophagy

Additional regulatory mechanisms

Skeletal muscle contributes approximately 40% of total body mass and therefore represents a key mediator of metabolic disease (Gallagher et al., 1998). Furthermore, skeletal muscle is composed of different fiber types with distinct morphological and physiological differences. Simply, skeletal muscle can be defined as type I (red), slow-twitch oxidative, or type II (white), fast-twitch glycolytic. A consideration when analyzing skeletal muscle autophagy is the type of muscle being investigated since recent evidence suggests that autophagy may respond to stimuli in a fiber type-specific manner. Ogata et al., (2010) reported a differential fasting-induced autophagic response

in red and white muscle of rats withheld food for 1, 2 or 3 days. The white plantaris muscle showed a significant increase in autophagic flux compared to red soleus muscle, determined by an increase in LC3II:LC3I ratio. Muscle atrophy was also notably greater in plantaris than soleus muscle. Regulatory explanations suggest that differences were independent of transcriptional changes indicated by similar total and phosphorylated levels of FoxO3a. Levels of total and phosphorylated Akt were also comparable between fiber types. Reduced phosphorylation of mTOR in plantaris muscle was apparent, which may explain differences in autophagic activity relative to soleus. The authors suggest that the difference in phospho-mTOR may be due to the constitutive weight bearing activity of soleus muscle, which promotes mTOR phosphorylation via activation of phospholipase D (Hornberger et al., 2006). Ogata and colleagues (2010) determined phospho-mTOR using serine 2448, a known phosphorylation site of Akt. This would support weight bearing activity-induced phosphorylation via phospholipase D due to similar Akt phosphorylation status between plantaris and soleus. However, whether mTOR is responsible for the reported differences in muscle fiber type autophagy and atrophy is not clear. By one estimate, based on analysis of several studies utilizing transgenic and pharmacological modulation of mTOR, only approximately 10% of autophagy regulation may be explained by mTOR in skeletal muscle (Neel, Lin and Pessin, 2013).

Similar results to those of Ogata et al., (2010) have been reported in white EDL (extensor digitorum longus) and soleus muscle of WT mice fasted for 30 hours (Yamada et al., 2012). The phosphorylation status of AMPK, Akt and the downstream mTORC1-dependent S6K1 were similar between EDL and soleus, suggesting a mechanism independent of those discussed above. Comparisons of protein levels under fasting and fed conditions between EDL and soleus muscle

indicated that decreased vps34, the class III PI3K and component of the Beclin-1 complex required for initiation of autophagosome formation, may mediate differences in autophagy between white and red skeletal muscle. Determination of vps34/Beclin-1 assembly through immunoprecipitation supported the conclusion. Phosphorylated STAT3, by Y705, also showed EDL-specific changes in response to fasting and feeding. The upstream mediator of these findings appears to be Fyn protein kinase, which showed a two-fold activity increase in fasted compared to fed EDL muscle, but remained unchanged in soleus. Fyn KO mice confirmed the presence of a Fyn/STAT3/vps34 signaling cascade.

Fluctuations in autophagic activity between fasting and fed conditions in skeletal muscle fiber types may be differentially regulated through a Fyn/STAT3/vps34 signaling pathway. Fyn is a member of the Src family of tyrosine kinases, which through interactions with LKB1 (liver kinase B1) has been implicated in fatty acid oxidation and insulin sensitivity (Yamada et al., 2010). However, its precise role and upstream hormonal regulation in autophagy within white skeletal muscle is unknown. Additionally, signaling pathways specific to red skeletal muscle remain to be elucidated, if present. It is worth noting therefore that not only may differences in autophagic activity exist between muscle fiber types, but the mediating signaling pathways may differ from the canonical mechanisms discussed above. Reasons for such fiber type-dependent responses beyond that of postural support discussed earlier are not clear. It is plausible that red muscle is preferentially preserved in conditions of nutrient scarcity due to the high mitochondrial content of oxidative fibers, capable of dramatically increasing fatty acid oxidation in response to fasting/starvation to meet energy demands. Meanwhile, fast-twitch white muscle fibers contain greater concentrations of the relevant myosin heavy chain isoforms compared to slow-twitch fibers

(Greising et al., 2012), which may provide a more significant source of AA in times of need. Combined, selective autophagy of fast-twitch glycolytic fibers, less suited for physiological conditions under which blood glucose is compromised, aids in the preservation of slow-twitch oxidative fibers, through the provision of AA.

Further regulatory mechanisms in skeletal muscle, although not necessarily limited to skeletal muscle, are epigenetic alterations. One such example is deacetylation, or the removal of acetyl groups from histone tails through histone deacetylases (HDACs). HDACs provide a means of transcriptional suppression since the removal of acetyl groups lead to the compacting of chromatin (Haberland, Montgomery and Olsen, 2009). Moresi et al., (2012) discovered that 40% of mice with skeletal muscle-specific KO of HDAC1 and HDAC2 died within one day of birth, while survivors developed progressive myopathy preceded by impaired autophagy flux and development of an increased oxidative metabolic phenotype. KO mice were found to have a blunted LC3II:LC3I ratio response to fasting, combined with an accumulation of p62, together indicating dysfunctional autophagy. Induction of several key autophagy genes, including *atg5* and *Atg7* in response to 48 hours fasting were also suppressed. The same mice, when compared to controls, shifted to a predominantly oxidative muscle fiber type and exhibited increased energy expenditure independent of physical activity. Upregulation of PGC1b (peroxisome proliferator-activated receptor gamma coactivator 1b) and *Acadm* (acyl coenzyme A dehydrogenase), compared to control mice, is representative a shift to oxidative metabolic pathways. Conversely, overexpression of HDAC1 and HDAC2 lead to increased autophagy flux and reductions in p62, however no data for oxidative metabolism was reported. Aside from progressive myopathy, it is possible that reduced skeletal muscle autophagy may confer oxidative metabolic advantages and

greater resting energy expenditure. However, relevant regulatory autophagy targets of HDAC1 and HDAC2 are unknown. HDACs are also not limited to deacetylation of histones tails and may act directly on proteins to adjust function or activity (Choudary et al., 2009). It is unclear therefore whether alterations in oxidative metabolism and fiber type were the result or cause of impaired autophagy, or mediated through another pathway.

Perhaps the more novel finding, and of particular interest to the current review, was the discovery that HDAC1/2 KO mice were rescued from myopathy and exhibited normalized autophagy following an 8 week 60% HFD (Moresi et al., 2012). Expression of HDAC1 and HDAC2 were not altered, although control and KO mice had greater HDAC3 expression. The authors also fed HFDs to pregnant mice from one-day post conception and reported no lethal side effects in HDAC 1 and 2 KO offspring. The consumption of a HFD therefore proved protective against the deleterious effects of HDAC KO. While the mechanism of action was not discerned, epigenetic alterations are an attractive suspect following the reports of HFD consumption during pregnancy.

Skeletal muscle autophagy and metabolism

Skeletal muscle is responsible for approximately 80% of whole-body insulin-mediated glucose utilization (Baron et al., 1988) and a significant portion of whole-body fatty acid oxidation (van Hall et al., 2002), thus representing an indispensable target for investigating the etiology of insulin resistance and metabolic disease. Autophagy KO models in the liver and pancreas indicate that insulin resistance may be related to impaired autophagy. As with other tissues, Atg7 KO models have also been generated for tissue specific analysis of autophagy in skeletal muscle metabolism and function. Similar to the autophagy deficient-induced myopathy and atrophy reported in

HDAC1 and 2 KO mice (Moresi et al., 2012), Masiero et al., (2009) discovered profound muscle atrophy in *Atg7* null mice associated with sarcomere disruption and mitochondrial abnormalities. Fibers were notably smaller and determined to possess a reduced force capacity even when corrected for size. Intriguingly, no differences between fiber types were noted. This suggests that ablation of *Atg7* dismantles the signaling pathway and/ or machinery regulating the preferential upregulation of autophagy in fast-twitch glycolytic fibers during fasting, compared to slow-twitch oxidative fibers that was previously reported in response to fasting-induced autophagy (Yamada et al., 2012; Ogata et al., 2010). While markers of oxidative stress were greater, likely due to mitochondrial defects, no energy imbalance was detected based on inactive AMPK. Likewise, S6 phosphorylation status was similar between control and KO mice under both fed and fasting conditions implying intact mTORC1 signaling, accompanied by no significant changes in Akt phosphorylation. Unfortunately, more definitive metabolic outcome measures were not investigated.

A second skeletal muscle-specific *Atg7* mouse KO study, led by Kim et al., (2013), focused on alterations to both local and systemic metabolic function. In line with previous research (Moresi et al., 2012; Masiero et al., 2009), muscle atrophy was enhanced, and fibers were reduced in size. Gastrocnemius muscle was used to determine differences in skeletal muscle phenotype. In contrast to previous findings (Masiero et al., 2009), no significant reductions in muscle function, as measured by acute exercise to exhaustion, were detected. However, the measured indices of muscle function differed, which may account for this discrepancy. Non muscle-specific anthropometric changes were also noted. KO mice had lower total fat mass with reductions in fat pad and adipocyte size. Comparison of *Atg7* and p62 expression, and LC3II:LC3I ratio in white

adipose tissue of both KO and control mice confirmed that the differences were not the result of altered adipose autophagy. Differences in fat mass were not accounted for by differences food intake, which remained consistent with that of controls whether adjusted for body weight or not. Energy expenditure, although not significant, did show a trending increase ($P=0.06$), which could not be explained by differences in locomotive activity. The effects of alterations in skeletal muscle autophagy may not therefore be locally confined.

Assessment of metabolic homeostasis and function found reduced fasting glucose and insulin, as well as increased glucose tolerance and insulin sensitivity in KO male mice relative to controls. Plasma lipid profiles were unchanged. The gender-specific nature of the whole-body metabolic improvements is not clear, but suggests that the relationship between skeletal muscle autophagy and metabolism may not be equally shared in male and female mice. When challenged with a standard 60% HFD for 13-weeks, both male and female Atg7 KO mice demonstrated resistance to HFD-induced metabolic syndrome. Whole-body insulin-stimulated glucose uptake, glycolysis and glycogenesis were greater in HFD fed KO animals, compared to HFD fed controls. Strikingly, while skeletal muscle accounted for a portion of the HFD resistance, through increased insulin-stimulated glucose uptake compared to HFD fed controls, hepatic and particularly adipose tissue adaptations were predominantly responsible. Lipogenic and β -oxidation gene expression was reduced and increased, respectively, in livers of KO mice. No changes in hepatic β -oxidation were present, but lipid accumulation was attenuated in tandem with markers of liver injury. In vivo β -oxidation was significantly increased in KO mice, but ex vivo analysis of tissues and gene expression data indicated that despite the absence of a functional autophagy pathway, skeletal muscle β -oxidation was unchanged. Examination of adipose tissues found increased rates of in

vivo β -oxidation were the result of upregulated β -oxidation in WAT and BAT. Furthermore, uncoupling protein 1 (UCP1) and PGC1 α gene expression were enhanced in WAT and BAT. In combination with these data and other brown-like adipocyte marker genes, a distinctive browning of WAT was indicated. Finally, BAT activity, determined by BAT glucose uptake, was increased as were body temperature and energy expenditure in HFD fed skeletal muscle-specific Atg7 KO mice compared to HFD fed controls. Unlike non-HFD fed animals, male and female HFD fed mice were similarly protected against a metabolic syndrome, due to increased peripheral glucose uptake and adipocyte thermogenesis fueled by greater rates of β -oxidation. Alterations in skeletal muscle autophagy may therefore have systemic consequences, which might be relevant when considering the development and progression of metabolic disease. The reasons for this are not clear, but suggest a potential endocrine effect, altered blood glucose and lipid levels resulting from skeletal muscle metabolic dysregulation, or an error in the muscle-specific KO model.

The third and final skeletal muscle Atg7 KO mouse model paper produced disagreeing anthropometric data (Wu et al., 2009). Mice lacking skeletal muscle Atg7 were not subject to reduced body size or muscle atrophy. The authors also noted that these null findings were consistent across the first year of life, a temporal observation not provided by previous skeletal muscle KO papers (Kim et al., 2013; Masiero et al., 2009). Western blot analysis confirmed the absence of Atg7 and an accumulation of p62. Additional analyses were not reported, thus it is not possible to discern the cause of controversy. The scope of investigation by Wu et al., (2009) was limited, particularly with regard to skeletal muscle metabolic adaptations, but did find an accumulation of mitochondria characterized by morphological and functional abnormalities. These findings will be the focus of the second half of the review. Although Wu et al., (2009) did provide

comment on the condition of Atg7 KO mice one year into life, the long long-term consequences of autophagy deficiency seem under reported, and studies lack positive controls. The potential drawbacks of using KO models to investigate autophagy were elaborated earlier. Relationships between HFDs and autophagy drawn from such models may not therefore be reflective of real-world outcomes.

The effects of a HFD on skeletal muscle autophagy

The mouse model studies described above characterized the effects of autophagy deficiency on metabolism, which may be important to understand in the context of metabolic disease development. However, systems of intact autophagy are required to understand how HFDs affect skeletal muscle autophagy. Investigations into the influence of HFDs (Park et al., 2014; Koga et al., 2010; Yang et al., 2010; Ebato et al., 2008) or FFA exposure (Park et al., 2014; Lupi et al., 2002) in models of intact autophagy, in non-skeletal muscle tissues, previously discussed, suggest that autophagy activity is responsive to, and dysregulated by excessive fat exposure. He et al., (2012) described similar patterns of dysregulation in skeletal muscle of wild-type C57BL/6 mice following a HFD. Mice were fed a HFD for 12 weeks, following which baseline autophagy was significantly, albeit modestly, suppressed based on p62 accumulation, indicative of reduced autophagosome degradation. Autophagic activity, determined by LC3II:LC3I ratio, was not significantly changed. These data would suggest that HFD-induced suppression of autophagy occurs downstream of LC3 lipidation and may be linked to an error in autophagosome maturation, p62 sequestration of cargo or lysosome fusion. Deficient fusion between autophagosomes and lysosomes is an appealing mechanism of dysfunction due to previously discussed findings in hepatocytes (Park et al., 2014) and livers of HFD fed mice (Koga et al., 2010).

Consumption of a HFD is associated with ectopic lipid deposition in skeletal muscle and other non-adipose tissues (Hulver et al., 2003; Turpin et al., 2009). Lipid overload in non-adipose tissue, termed lipotoxicity (Unger, 2003) is associated with cellular dysfunction and increased apoptosis (Turpin et al., 2006; Bonnard et al., 2008; Koves et al., 2008). Autophagy provides a defense mechanism against apoptosis (Rikka et al., 2011), so increased apoptosis may be indicative of lipotoxic suppression of autophagy and subsequent loss of protection against apoptosis. Examination of HFD-induced lipotoxicity in mice provided evidence to the contrary (Turpin et al., 2009). Markers of apoptosis, including caspase 3 activity and Bcl-2:Bax ratio were unchanged following a HFD. Transcription of pro-apoptotic proteins with mitochondrial and nuclear associations were suppressed in response to a HFD. Furthermore, unchanged apoptosis in lipotoxic skeletal muscle is supported by similar findings in ob/ob and adipose triacylglyceride lipase (ATGL) null mice, both models of lipotoxicity (Turpin et al., 2009).

Turpin and colleagues (2009) considered whether HFD-induced skeletal muscle lipotoxicity might have regulatory consequences on autophagy. Eight week old C57BL6/J mice were fed a 45% HFD for 12 weeks. Skeletal muscle triacylglyceride (TAG) and diacylglyceride (DAG) content, determined by histological staining, were significantly increased, as was liver TAG, plasma insulin and fat mass. The LC3II:LC3I ratio, FoxO1 gene expression, mTOR gene expression or activity, determined by S6 kinase phosphorylation, were unchanged in HFD mice compared to chow fed controls. Beclin-1 mRNA was unchanged, although Bcl-2 protein levels were increased leading to the suggestion that Beclin-1 induction, and thus autophagosome formation, was absent due to the inhibitory binding of Bcl-2 to Beclin-1. While the authors concluded that lipotoxicity was not a

factor in regulating skeletal muscle autophagy, the supporting data suggest this might be premature. The LC3II:LC3I ratio is indicative of autophagic flux, but as previously discussed, HFD-induced dysregulation of autophagy may occur downstream of LC3I lipidation (He et al., 2012). An accompanying measurement of p62 accumulation would have provided a means of assessing autophagosome function, but was not reported. The utilization of FoxO1 gene expression to assess transcriptional regulation of autophagy is likely also inappropriate. There is concurrency in the literature that transcriptional regulation of skeletal muscle autophagy is under the control of FoxO3a (Sanchez et al., 2012; Mammucari et al., 2007 and 2008; Zhao et al., 2007). It is noted that FoxO1 has been reported to associate with Atg7 during autophagy induction within cancer cell lines (Zhao et al., 2010) and to regulate skeletal muscle protein synthesis (Southgate et al., 2007) and atrophy (Kamei et al., 2004), but its role in skeletal muscle autophagy is unclear. Additionally, the Spearman's correlation coefficient between mRNA and protein levels is approximately 0.4 (Wilhelm et al., 2014), supporting the need for accompanying analysis of total protein. This principle can be similarly applied to Beclin-1, thus limiting conclusions drawn as to its relationship with Bcl-2, and consequently its induction. Finally, while previously discussed as a central regulator of autophagy, Neel and colleagues' (2013) predicated that mTOR may only be a minor regulator of skeletal muscle autophagy and as such may not be a suitable principle marker for autophagic control. This discrepancy may be due to mTOR's function as a nutrient sensor, particularly amino acids (Dunlop and Tee, 2013). Early studies focused on starvation as a model for decrypting autophagy pathways (Noda and Ohsumi, 1998; Scott, Schuldiner and Neufeld, 2004), which may have over-emphasized the role of mTOR in autophagy. Basal autophagy and stimulated autophagy states such as oxidative stress may be regulated through alternative pathways, discussed later. The null findings by Turpin's group (2009) may also have been due to

the diet used. Previous studies investigating HFDs and autophagy have employed diets containing 60% fat (He et al., 2012; Kim et al., 2013; Singh et al., 2009; Zhang et al., 2009; Ebato et al., 2008), compared to 45% used by Turpin et al., (2009). Alterations to autophagy activity may then require a more substantial lipid overload. It is unclear though whether such HFDs can be considered physiologically relevant for humans in light of recent dietary data for US adults, suggesting that dietary fat accounts for approximately 33% of total calories consumed, with 10.6% of total calorie consumption attributed to saturated fat (National Center for Health Statistics). These figures do not however account for the total number of calories consumed or analyze fat intake based on overweight, obese or other metabolic disease states. Yang et al., (2010) found a potentially more physiologically relevant 35.5% HFD was still sufficient to markedly reduce hepatic indicators of autophagy in mice, including LC3, Beclin1, Atg5 and 7 protein levels. The effect of a HFD on skeletal muscle autophagy remains inconclusive.

Skeletal muscle autophagy and exercise

Exercise has been known to initiate autophagy in skeletal muscle of mice for three decades (Salminen and Vihko, 1984), however, only in recent years have investigators learned that autophagy is a key mediator of exercise-induced adaptation. Exercise results in significant skeletal muscle remodeling, including the synthesis of contractile proteins and key enzymes, angiogenesis and mitochondrial biogenesis. These adaptations lead to increased insulin sensitivity and improved glucose homeostasis, aiding the prevention and management of obesity and T2D as well as increasing all-cause mortality. Acute bouts of treadmill exercise have been found to initiate skeletal muscle autophagy in mice (Grumati et al., 2011; Jamart et al., 2013). It is likely that this increase is at least in part due changes in cellular energy status i.e. a shift in the AMP:ATP ratio,

indeed exercise-induced autophagy is exaggerated in fasting state exercise (Jamart et al., 2013). However, He et al., (2012) showed that insulin sensitivity, GLUT4 translocation and AMPK activity were attenuated in exercising mice lacking the capacity to induce exercise-stimulated autophagy using Bcl-2 AAA mice. Bcl-2 AAA mice, possess alanine residues in place of the exercise-stimulated phosphorylation sites, Thr 69, Ser 70 and Ser 84 (the homolog to human Ser 87) of Bcl-2. This impedes the exercise-stimulated dissociation of Beclin-1 from Bcl-2, thus preventing exercise-induced autophagy while maintaining basal autophagy. These mice, similar to wild-type controls, exhibited reduced basal autophagy in response to a HFD and were equally susceptible to HFD-induced obesity. Curiously, Bcl-2 AAA mice did not display improved metabolic outcomes in response to exercise, following and during a HFD. Wild-type controls improved glucose tolerance, reduced serum leptin, triglycerides and cholesterol and increased adiponectin in response to exercise. Bcl-2 AAA mice however did not show similar adaptations to exercise despite a similar attenuation of weight gain to controls. Exercise-induced autophagy is therefore an essential mediator of exercise related metabolic health outcomes, possibly through interactions with AMPK.

Lira et al., (2013) recently provided further evidence supporting autophagy as a mediator of exercise-induced adaptation. Firstly, the study found oxidative muscle exhibited higher rates of basal autophagy and autophagy proteins compared to glycolytic or mixed fiber muscles. Secondly, in response to exercise training, plantaris muscle, a mixed fiber muscle, increased autophagic flux and autophagy proteins while developing a more oxidative phenotype. PGC1 α over-expression mice, characterized by an expansive mitochondrial population and high oxidative capability, confirmed a correlation between increased autophagic flux and oxidative capacity. These mice

displayed increased LC3II:LC3I and reduced p62 accumulation providing evidence of increased non-stimulated (basal) autophagic flux because exercise was not undertaken. Increases in autophagy flux may therefore have been the result of enhanced oxidative capacity resulting from increased mitochondrial density but may also have been a direct response to higher mitochondrial content, not oxidative capacity. Finally, exercise training was discovered not to increase endurance capacity in transgenic mice with reduced Beclin-1 expression. This particular mouse model demonstrates undisrupted basal autophagy, but was discovered to have a limited ability to increase basal autophagy in response to exercise training. These data suggest that oxidative capacity is linked to autophagic flux and that exercise-stimulated increases in basal autophagy are required for exercise adaptation and improved performance. Mitochondrial content was not measured in the reduced Beclin-1 expression mice, however, the inability to improve endurance capacity and failure to upregulate PGC1a, a key transcription factor in mitochondrial biogenesis, may indicate that mitochondrial biogenesis and content were unchanged with training. It is possible therefore that the rate of basal autophagy regulates mitochondrial biogenesis and content. Of note, reduced Beclin-1 expression mice have a suppressed acute exercise-stimulated autophagy response, also associated with reduced exercise-induced AMPK activity (He et al., 2012), a factor not considered by the authors (Lira et al., 2013). Furthermore, the Beclin-1 reduction was not skeletal muscle specific, thus systemic effects need to be considered. The importance of basal autophagy in regulating adaptive improvements to endurance capacity following exercise training remains to be determined.

Despite strong evidence in favor, the literature is not unanimous that exercise stimulates autophagy. Kim and colleagues (2013) found no evidence for increased autophagy in response to

moderate exercise. The exercise protocol involved a 5° slope, which would have increased the eccentric load of the exercise bout, possibly confounding the results when compared to those of He et al., (2012) and Grumati et al., (2011), neither of which employed a slope. However, additional eccentric load would be expected to increase muscle damage (Proske and Morgan, 2001) and likely require upregulation of autophagy for the clearance of damaged proteins. This was not found to be the case even 12hrs post exercise. Differences in exercise duration and intensity between the studies, as well as poorly defined protocols make interpretation difficult. Regardless, the main purpose of the current review is to investigate human findings, which have greater clinical relevance.

Human skeletal muscle autophagy and exercise

Interest in autophagy and metabolic disease has grown significantly in recent years leading to increased understanding of autophagy within rodent metabolic regulation. To date, literature pertaining to human skeletal muscle autophagy in metabolic diseases of obesity, insulin resistance and diabetes, in response to obesogenic diets or exercise, is limited or non-existent. Systematic searches produced two papers analyzing skeletal muscle and autophagy, both in relation to ultra-endurance exercise and from the same group.

Ultra-endurance events are those lasting in excess of 6 hours (Zaryski and Smith, 2005). The studies of interest investigated skeletal muscle autophagy in 11 and 8 well-trained ultra-distance runners, responding to 24-hour treadmill (Jamart et al., 2012a) and 200km (Jamart et al., 2012b) running events, respectively. The mean time to complete the 200km race, completed at sea level, was 28 hours. Both studies analyzed muscle samples biopsied from the vastus lateralis, pre and

post event, and frozen for later analysis. It should be noted that the muscle biopsy samples analyzed by Jamart et al., (2012a and 2012b) were collected during the course of two previous studies conducted by Millet et al., (2011) and Kim et al., (2011), respectively. Jamart et al., (2012a) reported significant increases autophagy markers, conjugated atg5-atg12 and LC3BII, although atg3, responsible for LC3I lipidation showed no significant change and Atg7 was unaltered. In line with these findings, LC3B and atg12 gene transcription were significantly greater following a 200km race (Jamart et al., 2012b). Other autophagy markers, including atg4b, required for LC3 priming prior to PE conjugation (Reggiori and Klionsky, 2002), and the lysosomal enzyme cathepsin L were also transcriptionally upregulated (Jamart et al., 2012b). However, cathepsin L activity following 24-hour treadmill running was not significantly changed, but most likely due to high interindividual variation. In combination these autophagy markers indicate a transcriptional and translational shift in favor of skeletal muscle autophagy in response to ultra-distance running.

Protein expression of autophagy mediators, Beclin-1 and BNIP3, were not significantly changed in response to 24 hours of treadmill running (Jamart et al., 2012a). A similar response was found for Beclin-1 mRNA response to a 200km race, however, BNIP3 gene expression was significantly increased in tandem with its antagonist, BNIP3L. ULK2, a functionally redundant, although as of yet not fully characterized homolog of the autophagy mediator ULK1 (Lee and Tournier, 2011), showed no transcriptional alteration (Jamart et al., 2012b). These data are difficult to interpret because while no changes in protein expression were reported, the protein activity is unclear. For example, Beclin-1, although a mediator of autophagy, can only initiate formation of a nucleation complex once released from Bcl-2. Jamart et al., (2012a) undertook a further analysis of the regulatory signaling pathway, not conducted by Jamart and colleagues (2012b). The

phosphorylation state of Akt and downstream targets, FoxO3a and mTOR^{Ser2448}, was significantly reduced. mTOR^{Ser2481} phosphorylation was suppressed, but not significantly. A downstream target of mTOR, 4E-BP1, was also significantly dephosphorylated following 24 hours of treadmill running. Meanwhile, exercise-induced AMPK phosphorylation was significantly greater post-run. Signaling pathway protein phosphorylation status, and this autophagy activity, appears to be in accordance with autophagy marker data, suggestive of increased autophagy following cessation of ultra-endurance running in skeletal muscle. FoxO3a is the major skeletal muscle transcriptional regulator of autophagy proteins LC3 and BNIP3 (Mammucari et al., 2007). Dephosphorylation of FoxO3a, via Akt suppression, would allow translocation to the nucleus and upregulation of LC3 and BNIP3 mRNA as reported (Jamart et al., 2012b). Furthermore, reduced mTOR activity may allow for elimination of autophagy inhibition, although the extent of mTOR control in skeletal muscle seems limited (Neel, Lin and Pessin, 2013). In addition, mTOR blocking may signal a shift in the anabolic-catabolic balance represented by decreased activity of 4E-BP1 and increased LC3BII (Jamart et al., 2012a).

Limitations in the studies include biopsy timing and nutritional intake during exercise. Jamart et al., (2012a) administered a second biopsy within 10 minutes of completing 24 hours of treadmill running, while Jamart et al., (2012b) sampled muscle 3 hours post-race. Non-significant protein changes found by Jamart et al., (2012a) may therefore be a by-product of sample timing. The underlying causes of autophagy induction are also not entirely clear. Participants in both studies were provided food and water ad libitum and estimated to have consumed approximately 30% of energy demands from exogenous sources (Jamart et al., 2012a). Jamart et al., (2012b) did not measure caloric intake, but given the similar nature of the event an energy deficit can be assumed.

The resulting negative energy balance would be anticipated to increase autophagy. Markers relating to oxidative stress were not measured in either study, which may have provided a stimulus for autophagy. Also, given the limited understanding of skeletal muscle autophagy in response to different nutrients it is not possible to determine whether food intake during exercise was a confounding factor. Future research is required to understand the effects of chronic training and shorter duration exercise on skeletal muscle autophagy. In light of exercise and HFD findings in mice, already discussed, the influence of exercise training and HFDs on the autophagic response and basal autophagy in human participants should be examined. Finally, if altered, does autophagy provide a defensive mechanism against potentially dysregulatory HFDs in trained humans leading to protection against metabolic disease.

Mitochondria

Mitochondrial function and metabolic disease

Mitochondria are essential organelles responsible for the efficient provision of energy through the chemiosmotic process of oxidative phosphorylation (Kotiadis, Duchen and Osellame, 2014). The most basic definition for mitochondrial function therefore is the ability to appropriately adjust ATP production in response to changing energy demands (Brand and Nicholls, 2011). However, mitochondria perform numerous other tasks including the synthesis of important molecules (such as phospholipids and heme), replication of mtDNA and import of nuclear-encoded proteins, assistance in calcium regulation, ROS production and elimination, and pro-apoptotic protein sequestration (Gottlieb and Carreira, 2010; Ryan and Hoogenraad, 2007; Rizzuto and Pozzan, 2006).

Classic assessment of mitochondrial function involves measuring isolated mitochondrial oxygen consumption in the presence of ADP (state 3_{ADP} respiration), the ATP synthase inhibitor oligomycin (state 4_o respiration), and FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) for the determination of uncoupled respiration (state 3_u) (Brand and Nicholls, 2011). The ratios of state 3_{ADP} or state 3_u and state 4_o define the respiratory exchange ratio (RCR), a measure of mitochondrial quality and capacity for substrate oxidation and ATP turnover, accompanied by low proton leak (Brand and Nicholls, 2011). Additional markers of dysfunction include reduced enzyme activity, mtDNA deletions, increased markers of oxidation and morphological abnormalities (Brand and Nicholls, 2011; Hill et al., 2012).

Skeletal muscle from obese, insulin resistance and T2D patients is associated with reduced mitochondrial activity, content and genes regulating oxidative phosphorylation (summarized in table 1). The precise role of mitochondrial dysfunction in the development and progression of metabolic disease remains controversial (Boushel et al., 2007; Rabol et al., 2009). However, a growing body of evidence implicates HFDs, as a cause of compromised mitochondrial function in skeletal muscle (Bonnard et al., 2009; van der Broek et al., 2009; Jheng et al., 2012; Yuzefovych et al., 2013). Many of the maladapted mitochondrial function measures exhibited following a HFD, correlate to those found in skeletal muscle-specific Atg7 KO mice (Wu et al., 2009; Kim et al., 2013). Together, these findings suggest that a HFD may disturb mitochondrial quality control, via suppressed mitophagy, leading to an accumulation of dysfunctional mitochondria.

Mitochondrial quality control

The relationship between metabolic disease and mitochondrial function emphasizes the need for strict control of mitochondrial quality. The mechanisms regulating quality require monitoring and control of components, function, and products, as well as the communication between mitochondria and other cellular components (Kotiadis, Duchon and Osellame, 2014). While mitophagy is responsible for degradation of the whole organelle, mitochondria possess alternative means of controlling individual protein quality. Heat shock proteins (Hsp) such as Hsp60 and Hsp70 act as chaperones, directing the import and folding of ETC proteins, while preventing unfolded and misfolded protein aggregation (Pellegrino, Nargund and Haynes, 2013). Degradation of oxidized, dysfunctional or misfolded proteins is managed by a variety of proteases localized throughout a mitochondrion (Marcillat et al., 1988; Jung, Catalgol and Grune, 2009). Turnover of electron transport components, for example, rely on the matrix and inter-membrane space localized proteases m-AAA and i-AAA, respectively (Leonhard et al., 1996). Cytosol facing proteins localized to the OMM can be turned over independently of mitophagy via the ubiquitin-proteasome systems (Karbowski and Youle, 2011).

Table 1. Summary of studies analyzing human skeletal muscle mitochondrial function under pathologies of obesity, insulin resistance and type II diabetes. +, findings support mitochondrial dysfunction; -, findings do not support mitochondrial dysfunction.

Authors	+ or - finding	Population	Conclusion
Befroy et al., (2007)	+	Lean insulin-resistant offspring of type 2 diabetics	Reduced mitochondrial substrate oxidation – possibly inherited.
Boushel et al., (2007)	-	Type 2 diabetic elderly	Normal mitochondrial function. Blunted coupled and uncoupled respiration attribute to lower mitochondrial content.
Chanseume et al., (2010)	+	Overweight and insulin resistant obese	Insulin resistant obese males produce less ATP and ROS compared to lean controls. Overweight exhibit increased state 3 respiration. No differences in enzyme activity or mitochondrial content.
Hernandez-Alvarez et al., (2010)	+	Obese early onset type 2 diabetics	No changes in mitochondrial mass, but reductions in Mfn2 and ATP synthase.
Hwang et al., (2010)	+	Obese non-diabetic and type 2 diabetic – middle age	Reductions in mitochondrial proteins in type 2 diabetic skeletal muscle.
Kelley et al., (2002)	+	Obese non-diabetic and type 2 diabetic	Impaired bioenergetic capacity in obese and more so in type 2 diabetes, accompanied by reduced mitochondrial size
Kristensen et al., (2014)	+	Carriers of insulin receptor gene mutation	Inherited insulin resistance coincides with reduced mitochondrial oxidative capacity in a PGC1 α and muscle fiber type-specific fashion.
Mootha et al., (2003)	+	Type 2 diabetics	OXPHOS genes are coordinately downregulated in human
Morino et al., (2005)	+/-	Lean insulin resistant offspring of type 2 diabetics	Reduced mitochondrial content. *functional parameter not measured
Patti et al., (2003)	+	Type 2 diabetic and family history positive	Downregulation of OXPHOS genes in family history positive and particularly in type 2 diabetes. Downregulated in response to reduced expression of NRF-1 and PGC1 α found in type 2 diabetes and family history positive.
Petersen et al., (2004)	+	Lean insulin resistant offspring of type 2 diabetics	Reduction in OXPHOS activity possibly due to inherited defect.
Rabøl et al., (2009)	-	Type 2 diabetic middle aged overweight and obese	Mitochondrial respiration was lower in type 2 diabetes compared to controls, when there was no difference when normalized to citrate synthase. Mitochondrial content was reduced in type 2 diabetes.
Ritov et al., (2010)	+	Obese type 2 diabetics and obese	Increase citrate synthase activity in obese. NADH oxidase significantly reduced in obese and obese type 2 diabetics. No change in mitochondrial content.
van de Weijer et al., (2013)	+	Type 2 diabetic	Impaired PCr recovery indicative of mitochondrial dysfunction.
Vijgen et al., (2013)	+	Morbidly obese	Decreased state 3 respiration when normalized for mitochondrial content.
Wijngaarden et al., (2013)	+	Non-diabetic obese	Reduced respiratory chain content in non-diabetic obese.

A major component of mitochondrial quality control, beyond that of individual protein quality, and pertinent to the current review, is mitochondrial turnover. The maintenance of a healthy, functional network of mitochondria requires turnover through regulated shifts in the balance between mitochondrial biogenesis, mitophagic flux, and cycles of fission and fusion (Gottlieb and Carreira, 2010; Patel, Shirihai and Huang, 2013). It is perhaps surprising then that relatively little is known about mitochondrial turnover. In recent years, the discovery PGC1 α has led to an intensive focus on mitochondrial biogenesis, but relatively little with regard to mitophagy. Much of the available data on mitochondrial turnover was completed over 20 years ago, producing highly variable results (Gottlieb and Carreira, 2010). Early attempts to measure turnover in rats suggested that mitochondria may have a half-life of as little as 4-6 days in heart and liver (Rabinowitz and Zak, 1975; Lipsky and Pedersen, 1981), or as much as 10-25 days in the heart, liver and brain (Menzies and Gold, 1971; Pfeifer, 1978). Estimates for skeletal muscle are rare, but find a half-life of 7 days based on cytochrome c (Booth and Holloszy, 1977). A more recent study in the livers of mice reported mitochondrial half-life figures of 1.83 days or 1.16 days following 3 months calories restriction, providing evidence for increased mitochondrial turnover with calorie restriction (Miwa, Lawless and von Zqlinicki, 2008). Turnover rates are likely therefore tissue-specific. Currently, precise figures for the rates of mitochondrial turnover are unknown due to technical limitations (Gottlieb and Carreira, 2010). However, numerous markers and mediators of mitochondrial turnover are known and measurable. The use of such measures may therefore be employed to better understand the balance of these processes within the context of diet and exercise to progress understanding of turnover in the maintenance of mitochondrial quality control.

Mitochondrial dynamics

Mitochondria are dynamic organelles forming a constantly shifting tubular reticulum defined by frequent fission and fusion events (Twig et al., 2008). These events, termed mitochondrial dynamics, allow the sharing of membrane structures (Wikstrom, Twig and Shirihai, 2009), metabolites (Karbowski et al., 2004; Jakobs, 2006) and mtDNA (Nakada et al., 2001; Ono et al., 2001). The delicate balance between fission and fusion dictates mitochondrial network morphology and mitochondrion size (Benard and Karbowski, 2009). Morphology is highly variable between cell types, ranging from small fragmented networks to extensive elongated tubules (Detmer and Chan, 2007). Understanding about how fission and fusion are mediated has been aided by the discovery of several fission and fusion-specific proteins. Skeletal muscle mitochondria, specifically intermyofibrillar mitochondria, are uniquely arranged between myofilament bundles (Koves et al., 2005), resulting in limited mitochondrial motility (Liu et al., 2009) and thus doubt as to whether mitochondrial dynamics exists in skeletal muscle (Liu et al., 2014). Recently though was it was discovered that skeletal muscle mitochondrial populations are highly dynamic, adjusting mitochondrial morphology accordingly, in response to diet and exercise (Jheng et al., 2012; Iqbal et al., 2013; Liu et al., 2014). Investigation of skeletal muscle mitochondrial fission and fusion may therefore yield insights in to metabolic homeostasis and disease.

Mitochondrial fission

Mitochondrial fission machinery consists of several identified proteins, among which dynamin-related protein 1 (Drp1) is suspected to be of vital importance (Labrousse et al., 1999; Smirnova et al., 2001). Primarily located in the cytosol, Drp1 translocates to the OMM where it polymerizes

into multimeric ring complexes around mitochondria upon fission initiation (Legesse et al., 2003; Ingeman et al., 2005). GTP hydrolysis causes constriction of the rings and scission of the double membrane (Lackner, Horner and Nunnari et al., 2009; Mears et al., 2011). Four additional proteins of the OMM have been suggested as potential receptors for the recruitment of Drp1 to mitochondria (Otera et al., 2011; Losen et al., 2012). The role of human mitochondrial fission 1 protein (hFis1), a known OMM binding partner for mammalian Drp1 (Yoon et al., 2003), is controversial since reduced hFis1 expression does not prevent mitochondrial recruitment of Drp1 (Lee et al., 2004; Wasiak, Zunino and McBride, 2007). Studies investigating hFis1 have reported increased network fragmentation in response to hFis1 over expression, and greater network fusion with hFis1 knockdown (Yoon et al., 2003; Stojanovski et al., 2004). More recently, Otera et al., (2010) demonstrated no effect on network morphology when hFis1 was knocked down, and proposed that the previous RNAi based hFis1 knockdowns may have been invalid due off-target effects. Although the precise role of hFis1 is enigmatic, it remains a protein of interest within mediation and regulation of mitochondrial fission and function, respectively (Gomes and Scorrano, 2008; Loson et al., 2012).

A second OMM anchored protein, mitochondrial fission factor (Mff), represents a promising candidate for the role of Drp1 membrane receptor and self-assembly promoter (Otera et al., 2010; Loson et al., 2012). Knockdown of Mff in HeLa and MEF cell lines show increased network fusion and fewer mitochondrial localized Drp1 puncta, while Mff over-expression produces Drp1-dependent mitochondrial fragmentation (Otera et al., 2010; Loson et al., 2012). Mitochondrial dynamics proteins 49 kDa and 51 kDa (MiD49 and MiD51) represent additional components of the OMM fission machinery. In the absence of Fis1 and Mff, Drp1 can be recruited to mitochondria

via MiD49 and MiD51, both of which, when over-expressed or knocked down, disrupt mitochondrial network morphology (Otera et al., 2010; Palmer et al., 2011; Loson et al., 2012). A further Drp1-dependent protein, mitochondrial protein 18 kDa (MTP18), localized to the IMM produces similar mitochondrial network alterations to those previously discussed when over-expressed or knocked down (Tondera et al., 2005). Finally, the fatty acyl transferase, Endophilin B1, primarily located in the cytosol and operating downstream of Drp1 is necessary for the regulation of OMM dynamics during fission through lipid membrane remodeling (Karbowski, Jeong and Youle, 2004).

Regulation of mitochondrial fission

The regulation of fission is predominantly exercised through a series of post-translational modifications to Drp1, including phosphorylation (Chang and Blackstone et al., 2007; Taguchi et al., 2007; Cereghetti et al., 2008; Han et al., 2008), S-nitrosylation (Cho et al., 2009; Nakamura et al., 2010), ubiquitylation (Karbowski, Neutzner and Youle, 2007; Buhlman et al., 2014) and sumoylation (Zunino et al., 2009). The variety of regulatory levers implies a flexible system of mitochondrial fission management, capable of responding to a range of stimuli and stresses. Starvation-induced increases in cAMP, leading to the activation of PKA, causes an elongation of the mitochondrial network, resulting from Drp1^{Ser637} phosphorylation (Gomes, Benedetto and Scorrano, 2011). Suppression or pharmacological inhibition of mTOR produced a similar result, also mediated through PKA (Gomes, Benedetto and Scorrano, 2011). Arrested Drp1 trafficking following Drp1^{Ser637} phosphorylation leads to inhibited mitochondrial fission (Cereghetti et al., 2008; Gomes, Benedetto and Scorrano, 2011), although blocking of Drp1 GTPase effector activity may also contribute to fission prevention (Chang and Blackstone, 2007). Calcineurin acts

antagonistically to the effects of cAMP-PKA, dephosphorylating Drp1^{Ser637} in response to Ca²⁺, promoting Drp1 translocation and mitochondrial fission (Cereghetti et al., 2008). Mitochondrial depolarization stimulates Ca²⁺ release and calcineurin activation, followed by Drp1-activated fission, suggesting that calcineurin is a regulator of stress-induced mitochondrial division (Cereghetti et al., 2008). Similarly, Ca²⁺ activation of calmodulin-dependent protein kinase Ialpha (CaMK 1 α) increases mitochondrial fission via Drp1^{Ser600} isoform 3 phosphorylation (equivalent to Ser637 of isoform 1), augmenting Drp1 translocation and affinity for Fis1 (Han et al., 2008). During periods of mitosis, Drp1 is activated through phosphorylation of its Ser616 site via cyclin-dependent kinase 1 (Cdk1)/ cyclin B (Knott et al., 2008).

The turnover of Drp1 is regulated through interactions with the ubiquitin ligases MARCH5 and Parkin (Karbowski, Neutzner and Youle, 2007; Buhlman et al., 2014). Both MARCH5 and Parkin positively regulate Drp1, promoting mitochondrial fragmentation. MARCH5 purportedly acts to regulate Drp1 through the assembly and disassembly of fission complexes at sites of division (Karbowski, Neutzner and Youle, 2007). The precise role of Parkin-mediated fission is not clear due to its multiple OMM protein interactions (Chan et al., 2011; Wang et al., 2011), but is dependent on Drp1 interactions downstream of Ca²⁺-induced calmodulin/ calcineurin signaling (Buhlman et al., 2014). Nitric oxide is a versatile signaling molecule associated with Ca²⁺ control, oxidative stress and ETC suppression (Beltran et al., 2000; Buravlev et al., 2014), that S-nitrosylates Drp1^{Cys644} causing increased GTPase activity, and mitochondrial fission (Cho et al., 2009).

Mitochondrial fusion

Mitochondrial fusion requires amalgam of the IMM and OMM through coordinated action of the fusion machinery (van der Bliek, Shen and Kawajiri, 2013). Two types of fusion operate, the first being complete fusion, and the second transient, which entails division shortly after fusing, and aids in bioenergetic upkeep (Liu et al., 2009). Central to fusion are the OMM GTPases mitofusins 1 and 2 (Mfn1 and Mfn2), and the IMM GTPase optic atrophy 1 (OPA1) (van der Bliek, Shen and Kawajiri, 2013). Similar to the mitochondrial fission proteins, loss of functional Mfn1, Mfn2 or OPA1 leads to an opposing shift in dynamics, causing mitochondrial fragmentation (Chen et al., 2003; Karbowski et al., 2004). While Mfn1 and Mfn2 have complimentary functions (Palmer et al., 2011b), they also elicit distinct mitochondrial network morphologies when lost, implying additional independent roles in fusion (Chen et al., 2003). In support of this, Mfn1 was found to mediate the OMM tethering of fusion bound mitochondria in a GTPase hydrolysis-dependent manner (Ishihara, Eura and Mihara, 2004). Mfn2 is a multi-purpose protein associated with endoplasmic reticulum tethering and cell metabolism, which may regulate fusion complex assembly (de Brito and Scorrano, 2008; Benard and Karbowski, 2009; Sebastian et al., 2012).

The IMM fusion mediator, OPA1, governs cristae remodeling and inner membrane fusion (Ishihara et al., 2006; Liu et al., 2009). OPA1 is sensitive to changes in membrane potential, which regulates the ratio of fusion-competent long OPA1 isoform (L-OPA1) and fusion-incompetent short OPA1 isoform (S-OPA1) to determine IMM remodeling (Ishihara et al., 2006). In the presence of GTP and intact membrane potential, L-OPA1 oligomerises to merge the inner membranes of fusing mitochondria (Palmer et al., 2011b). Upon membrane depolarization L-

OPA1 is cleaved to S-OPA1 via matrix proteases in tandem with Mfn1 and Mfn2 degradation, leading to network fragmentation (Ishihara et al., 2006; Leboucher et al., 2012).

Regulation of mitochondrial fusion

Mitofusins 1 and 2 are regulated through several pathways. Mitofusin binding protein negatively regulates fusion through Mfn1 and Mfn2 interactions, although the mechanism is unknown (Eura et al., 2006). Bax and Bak, traditionally pro-apoptotic proteins, may be required for optimal Mfn2 function during fusion on health, non-apoptotic mitochondria through membrane transport assembly of Mfn2 (Karbowski et al., 2006). Following mitochondrial depolarization, mitochondrial dynamics shift to a fission cycle aided by the degradation of Mfn1 and Mfn2 through Parkin-mediate degradation (Gegg et al., 2010; Poole et al., 2010; Glauser et al., 2011). Depolarization also triggers OPA1 cleavage, specifically L-OPA1, through a series matrix localized proteases that regulate the ratio of OPA1 isoforms (Palmer et al., 2011b). The presenilin associated rhomboid-like protease (PARL) for example, is required for cristae maintenance, which becomes deformed when PARL is absent due to reduced availability of S-OPA1 (Cipolat et al., 2006). Fusion is also regulated through mitochondrial membrane lipids such as OMM cardiolipin. Mitochondrial phospholipase D (MitoPLD) promotes Mfn1 and 2-dependent fusion through the hydrolysis of cardiolipin to phosphatidic acid (PA) (Choi et al., 2006). The pro-fusion activity of PA is a regulator of transient fusion (Huang et al., 2001). Mitochondria undergo transient or “kiss and run” fusion events, which entail a rapid sequence of fusion and fission (Liu et al., 2009). These events appear to be regulated through the pro-fusion activity of phosphatidic acid (PA) and pro-fission activity of diacylglycerals (DAGs), which are generated through the dephosphorylation of PA (Huang et al., 2011).

Mitochondrial biogenesis

Biogenesis represents an essential balance to mitophagy within the mitochondrial quality control axis, maintaining total mitochondrial content, but also capable of increasing the population in response to dietary and exercise triggers (Irrcher et al., 2003; van den Broek et al., 2009). A detailed review of the underpinning biogenesis machinery and regulatory processes is outside the scope of the current review, although a brief description is provided below.

Synthesis of new mitochondria is not a de novo process, but a compliment of new proteins and pre-existing sub-compartments (Ryan and Hoogenraad, 2007). Mitochondria are a unique organelle owing to the division of DNA between the nucleus and mitochondrial matrix (Ryan and Hoogenraad, 2007). This segregation of DNA necessitates that unfolded proteins traverse the outer and inner membranes through specific translocases, namely, translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM). Upon entry to the matrix, proteins undergo folding via a series of matrix residing chaperones, critical to mitochondrial quality control (Ryan and Hoogenraad, 2007).

There are several key regulatory factors responsible for the coordinated transcription and translation of mitochondrial and nuclear derived DNA. Transcription factor nuclear respiratory factor 1 (NRF1) has been suggested to integrate biogenesis with mitochondrial function due to its control over genes involved in both (Ryan and Hoogenraad, 2007). One such gene, mitochondrial transcription factor A (Tfam), initiates mtDNA transcription, along with several additional transcription factors for nuclear-encoded proteins (Kelley and Scarpulla, 2004). However, these transcription factors do not account for the complex co-ordination of approximately 1500

mitochondrial proteins (Calvo et al., 2006). This has led to the extensive study of PGC1 α (Fernandez-Marcos and Auwerx, 2011), considered to be a universal regulator of vertebrate mitochondrial biogenesis (Ryan and Hoogenraad, 2007). A further regulator of mitochondrial mass is the mitochondrial DNA absence sensitive factor (MIDAS), which stimulates cardiolipin synthesis and regulates total mitochondrial lipid content, to affect mass independent of mitochondrial DNA, RNA or proteins (Nakashima-Kamimura et al., 2005).

Skeletal muscle PGC1 α and Tfam gene expression has been demonstrated to increase in response to 6 months calorie restriction in healthy, overweight individuals, in line with greater mitochondrial content (Civitarese et al., 2007), and to intermittent aerobic exercise in physically active males (Popov et al., 2014). Conversely, skeletal muscle from T2D patients show reduced PGC1 α and Tfam expression, and no response to 3 months aerobic training, while NRF1 was decreased (Chung et al., 2012). Similarly, cardiolipin content and structure is altered in conditions of T2D (Han et al., 2007) and diet-induced obesity (Faber et al., 2014). Together, these studies demonstrate a potential role for mitochondrial biogenesis regulators in mitochondrial quality control. It is interesting therefore that Parkin, a cytosolic protein that translocates to mitochondria during periods of mitophagy (Kubli and Gustafsson, 2012), similarly translocates to mitochondria during a proliferation state (Kuroda et al., 2006). Upon mitochondrial translocation, Parkin leads to increased mtDNA transcription through an undefined association with Tfam (Kuroda et al., 2006), integrating mitochondrial biogenesis with mitophagy.

Mitophagy

Clark (1957) was first to discover the sequestration of mammalian mitochondria within an autophagosome. However, until relatively recently this has been considered a random engulfing of cytosolic components rather than a selective event (Deter and De Duve, 1967; Kim et al., 2001). It is now well understood that mitochondria undergo selective degradation through a process termed mitophagy (Lemasters, 2005). It is necessary to distinguish between basal and acute stress-induced mitophagy. Basal mitophagy refers to the maintenance of cellular homeostasis, removing superfluous mitochondria and preventing the accumulation of dysfunctional mitochondria (Kubli and Gustafsson, 2012). Stress-induced mitophagy is purposed with promoting cell survival in response to specific insults by removing damaged mitochondria prior to apoptosis initiation (Kubli and Gustafsson, 2012). Mitophagy requires lysosomal degradation and thus shares much of the same molecular machinery as general autophagy, but depends upon selective mechanisms for the targeted elimination of damaged or dysfunctional mitochondria (Lemasters, 2005; Kim, Rodriguez-Enriquez and Lemasters, 2007; Soubannier et al., 2012; Lemasters, 2014). While current understanding of how such specificity is conferred to mitochondria, at least in mammalian cells, remains elementary (Goldman et al., 2010; Redmann et al., 2014), a series of candidate mechanisms and proteins have recently been identified.

Mechanisms of targeting mitochondria for elimination

An emerging mechanism for the selective nature of mitophagy lies in mitochondrial membrane potential, an indices of mitochondrial function (Hill et al., 2012). As previously discussed, depolarization can trigger fission and therefore represents a mechanism of separation from the mitochondrial reticulum. Fission generates a sub-population of these depolarized mitochondria

that do not undergo further fusion and thus remain segregated from the mitochondrial network (Twig et al., 2008). Studies in mammalian cells suggest that the fate of this depolarized sub-population is envelopment within autophagosomes and degradation (Elmore et al., 2001; Schweers et al., 2007; Narendra et al., 2008; Sandoval et al., 2008; Twig et al., 2008; Suen et al., 2010). The PINK1/ Parkin pathway appears to be a likely mediator between mitochondrial isolation and recruitment to the autophagosome.

Mitochondrial depolarization triggers the translocation and docking of the ubiquitin ligase, Parkin, from the cytosol to the outer mitochondrial membrane (OMM) (Narendra et al., 2008; Suen et al., 2010) via voltage-dependent anion channels (VDACs) (Geisler et al., 2010; Sun et al., 2012), in a PINK1-dependent manner (Matsuda et al., 2010; Narendra et al., 2010). Parkin is known to ubiquitinate MFN1 and 2 (Gegg et al., 2010; Poole et al., 2010; Glauser et al., 2011) and mitochondrial Rho-GTPase (MIRO) (Wang et al., 2011; Liu et al., 2012) for proteosomal degradation. These proteins are involved in mitochondrial fusion and motility, suggesting that Parkin may assist in the isolation of depolarized mitochondria. While the PINK1-dependent nature of Parkin recruitment is disputed (Dagda et al., 2009), PINK1 accumulation on the OMM is membrane potential-dependent. Under conditions of intact mitochondrial membrane potential PINK1 is imported and cleaved in the mitochondrial matrix by the protease Lon (Thomas et al., 2014). Upon depolarization PINK1 accumulates at the OMM (Kubli and Gustafsson, 2012), activating Parkin through ubiquitin phosphorylation (Kane et al., 2014). Translocation of cytosolic Parkin to the OMM is also mediated by BNIP3 (Lee et al., 2011), a known autophagy-inducer (Hamacher-Brady et al., 2007; Quinsay et al., 2010; Rikka et al., 2011) that stimulates Drp1-

dependent mitochondrial fission and MFN1 degradation (Lee et al., 2011). These processes seem to provide a means of mitochondrial tagging for selective elimination.

Several mechanisms for the removal of tagged mitochondria through autophagosome engulfment exist (Kubli and Gustafsson, 2012; Strappazzon et al., 2014). The first recruits Parkin following membrane depolarization, which initiates polyubiquitination of OMM proteins (Pankiv et al., 2007; Kirkin et al., 2009; Lamark et al., 2009; Kubli and Gustafsson, 2012). The ubiquitin-associated domain provides a docking site for p62, allowing the anchoring of mitophagy-tagged mitochondria to autophagosomes through its LC3-interacting region (Seibenhener et al., 2004; Pankiv et al., 2007). The generation of autophagosomes first requires stimulation of the initiation complex via PI3K, downstream of Bcl-2-bound AMBRA1 and localized to mitochondria (Strappazzon et al., 2011). Parkin has been found to interact with AMBRA1, an interaction increased in response to depolarization, a triggering event for both Parkin (Narendra et al., 2008; Suen et al., 2010) and AMBRA1 recruitment (Van Humbeeck et al., 2011, Strappazzon et al., 2014). Autophagosome engulfment and lysosomal fusion ensues, leading to mitochondrial clearance. A separate ubiquitin-independent pathway has also been proposed. BNIP3 and NIX, act as mitophagy receptor proteins, upon recruitment to mitochondria, binding directly to lipidated LC3, through LC3-interacting regions, tethering tagged mitochondria to autophagosomes (Kanki, 2010; Hana et al., 2012). Finally, a recent study reported a third potential pathway of mitochondrial tagging and removal via AMBRA1-induced mitochondrial depolarization and LC3 binding, independent of Parkin and p62 (Strappazzon et al., 2014). The mechanisms are not currently well defined.

Regulation of mitophagy

These pathways may provide mechanisms for regulating the stress-induced acute response and basal mitophagy, although it remains unclear as to which specific events preferentially trigger acute or basal mitophagy. The Parkin-ubiquitin-dependent pathway is a likely candidate for acute stress-induced mitophagy. Mitochondrial depolarization can be induced by oxidative stress (Vayssier-Taussat et al., 2002; Maharjan et al., 2014), providing a link to PINK1/ Parkin activation and subsequent polyubiquitylation, and p62-LC3 autophagosome anchoring. BNIP3, however, can initiate mitophagy independent of depolarization and other triggers, including ROS, Ca²⁺ and mitochondrial permeability transition pore (mPTP) (Quinsay et al., 2010; Rikka et al., 2011). Rikka et al., (2011) found BNIP3 was capable of inducing mitochondrial dysfunction, confirmed by impaired ATP production resulting from BNIP3-dependent reductions in electron transport chain complexes, which stimulated mitochondrial turnover. Reductions were not the result of impaired transcription or translation of either nuclear or mitochondria encoded subunits, but due to increased mitochondrial protease activity (Rikka et al., 2011). BNIP3 may therefore be capable of stimulating protease and autophagy regulated mitochondrial quality control, seemingly absent of cellular stress or externally-induced dysfunction. Nix is reportedly involved in mitophagy during erythrocyte maturation (Kanki et al., 2010). This may suggest BNIP3/ NIX-regulated mitophagy as a mechanism for basal/ housekeeping mitophagy.

Transcriptional regulation of Parkin and PINK1 are under the control of p53 (Zhang et al., 2011) and FoxO3a (Mei et al., 2009), respectively. A notable tumor suppressor protein involved in glucose metabolism and anti-oxidant defense, p53, increases Parkin transcription in response to H₂O₂ and under basal conditions (Zhang et al., 2011). A negative feedback loop between Parkin

and p53 appears to exist through direct binding of Parkin to the p53 promoter region (da Costa et al., 2009). Zhang and colleagues (2011) suggested that Parkin mediates the metabolic effects of p53, enhancing mitochondrial OXPHOS and reducing ROS through elevated reduced glutathione concentrations. It is not clear if Parkin acts directly on glutathione or if elevated concentrations are an indirect result of ROS suppression. Interestingly, PINK1 is also transcriptionally activated in response to oxidative stress via FoxO3a, and thus negatively regulated by PI3K/ Akt (Unoki and Nakamura, 2001; Mei et al., 2009; Priyadarshini, Orosco and Panula, 2013). Similarly to its mitophagy-inducing partner, Parkin, PINK1 is also suggested to increase reduced glutathione concentrations (Mei et al., 2009).

In addition to stimulating transcription of both Parkin and PINK1, ROS also sensitize the translocation of Parkin to mitochondria (Joselin et al., 2012). However, excessive ROS instigates defective PINK1-Parkin signaling (Whitworth et al., 2005). Parkin, through its cysteine side chains, is susceptible to s-nitrosylation and oxidation from ROS, leading to a loss of function (Winklhofer et al., 2003; Chung et al., 2004; Yao et al., 2004, Meng et al., 2011). The Parkin response to free radical stress may be biphasic, characterized by an initial increase in activity followed by a subsequent reduction upon continuous exposure (Yao et al., 2004; Meng et al., 2011). Consequently, mitochondrial quality control may be arrested, leading to the accumulation of depolarized and fragmented mitochondria (Youle and Narendra, 2011; Pilsel and Winklhofer, 2012). It may be relevant therefore to note that cell cultured Parkin knock-down models demonstrate increased glycolysis and lactate, in tandem with reduced pyruvate dehydrogenase activity and increased ROS production, while Parkin overexpression produced opposing effects (Zhang et al., 2011). Recently, Drew et al., (2014) corroborated these findings in myotubes

cultured from Parkin KO mice. The absence of Parkin caused elevated basal glucose uptake, but blunted insulin-stimulated glucose absorption. Drew et al., (2014) further demonstrated that HSP72 recruitment preceded the translocation of Parkin to depolarized mitochondria. HSP72 KO mice, exhibiting impaired Parkin recruitment, developed swollen dysmorphic mitochondria accompanied by lipotoxicity, reduced skeletal muscle respiratory capacity, and skeletal muscle insulin resistance (Drew et al., 2014). Interactions between Parkin and HSPs have previously been associated with the turnover of mitochondrial proteins through proteosomal degradation, rather than the whole organelle (Kirkin et al., 2009). However, in response to starvation HSP72 KO mice, relative to controls, did not stimulate LC3 cleavage, required for autophagosome tethering, demonstrating that autophagy was impaired. Together, these findings lend significance to the notion that the PINK1/Parkin pathway defends mitochondrial quality in response to acute oxidative stress, while chronic exposure may impair mitophagy-induction producing aberrant mitochondria and metabolic disease.

Regulation of BNIP3 has not been clearly defined due to conflicting results and various functions mediated through multiple cellular pathways (Vasagiri and Kutala, 2014). Notably, BNIP3 is under transcriptional regulation of FoxO3a, which in turn requires phosphorylation via p38 β , and is independent of AMPK (Mammucari et al., 2007; Lin et al., 2014). FoxO3a and BNIP3 also work in concert, through a BNIP3/ Rheb interaction, to suppress mTORC1 during periods of fasting (Lin et al., 2014). Akt, a known suppressor of FoxO3a, also inhibits BNIP3 transcription, although NF- κ B is the regulating transcription factor, not FoxO3a (Shaw et al., 2008). Epigenetic alterations under oxidative stress have a stimulatory effect on BNIP3 (Naushad et al., 2012; Lakshmi et al., 2013), which may function as a cellular redox sensor (Kubli et al., 2008). Such a sensor might

merge metabolic stress and mitophagy through enhanced BNIP3 activity. Naushad et al., (2014) reported that the BNIP3 gene exhibited hypomethylation in obese patients, leading to suppressed BNIP3 activity. This is of particular interest to the current review following work by Glick and colleagues (2012) who discovered BNIP3 KO murine hepatocytes demonstrated reduced β -oxidation, and increased lipid accumulation and de novo fatty acid synthesis. Perhaps somewhat counter intuitively, BNIP3 KO increased insulin sensitivity and glycolysis. Mitochondria content was also increased, but they were morphologically abnormal with a reduced membrane potential. The authors, however, concluded that the increase in dysfunctional mitochondria was due to a loss of BNIP3-dependent mitophagy. The significance of greater insulin sensitivity was not clear, but may be an artifact of in vitro experiments with murine cells commonly exposed to a high glucose environment. While data suggests that regulation of mitophagy may have metabolic consequences, it also highlights the dearth in literature characterizing mitophagy in human participants, particularly skeletal muscle.

Integrating mitochondrial bioenergetics, dynamics and mitophagy

The integration of mitochondrial dynamic processes and mitophagy for the efficient removal or rescue of mitochondria is well documented. Downregulation of Drp1 increases network elongation, preventing mitochondrial fission and mitophagy, resulting in impaired function and an accumulation of mtDNA-deficient mitochondria with increased oxidized proteins (Parone et al., 2008; Twig et al., 2008). Overexpression of OPA1 attenuates mitophagy and promotes dysfunction, although reports conflict as to whether the morphological shift is one of greater fusion or fission (Cipolat et al., 2004; Griparic et al., 2004; Chen et al., 2005; Twig et al., 2008). Recent work investigating the relationship between bioenergetics, dynamics and mitophagy, elegantly

demonstrated how the three systems interact. Melser et al., (2013) grew human skeletal muscle cells in glucose-containing or glutamine-containing media to manipulate cells into a state of low or high OXPHOS status, respectively. High OXPHOS cells were bioenergetically more efficient, produced more ROS and were subject to increased rates of basal mitophagy compared to low OXPHOS/ glycolytic cells. Upregulation of mitophagy was mediated through Rheb-dependent inhibition of mTORC1, which collaborates with BNIP3 (Lin et al., 2014), and associated with increased Nix-bound mitochondria. These findings support BNIP3 as a marker of basal mitophagy as previously discussed. The authors attributed the increase in basal mitophagy to local hypoxia, to which Nix and BNIP3 are sensitive (Bellot et al., 2009), resulting from increased oxygen consumption in response to the high OXPHOS cell status (Melser et al., 2013), providing a potential mechanism of exercise-induced mitophagy. Similar findings were reported in PGC1 α overexpression mice, in which basal mitophagy, determined by increased BNIP3, was found to be sensitive to oxidative capacity (Lira et al., 2013).

A similar experiment in retinal pigment epithelial (RPE1) cells found OXPHOS-dependent cells were resistant to fission, resulting from a failure to recruit Drp1 and cleave L-OPA1 (MacVicar and Lane, 2014). OXPHOS-dependent cells exhibited an elongated mitochondrial network, but without changes in the rate of basal mitophagy. However, increased OXPHOS activity did impair Parkin-mediated mitophagy (MacVicar and Lane, 2014), supported by a previous study that found Parkin recruitment was blunted in depolarized OXPHOS-dependent neurons (Van Laar et al., 2011). Together these studies suggest that both basal and stress-induced mitophagy may be altered in response to increased OXPHOS activity. The confounding reports on basal mitophagy and OXPHOS-dependence are not clear, but may relate to the difference in cell type used. Skeletal

muscle mitochondria possess a high bioenergetic efficiency relative to other tissue types (Marcinek et al., 2004), which in light of the above findings may give rise to bespoke mitophagic regulation.

Mitochondrial bioenergetic efficiency and/ or capacity can adapt to alterations in nutrient supply and demand (Liesa and Shirihai, 2013). This review has previously highlighted that impaired mitochondrial bioenergetics and dynamics are associated with states of metabolic disease (*see* table 1). The studies discussed above suggest that bioenergetics may govern mitochondrial dynamics and mitophagy activity. Maladapted bioenergetics may then initiate a degenerative quality control axis and a cycle of progressive dysfunction, leading to the development of metabolic disease. A greater understanding of how environmental cues affect mitochondrial quality control may therefore improve knowledge about the intricate relationship between mitochondria and metabolic disease.

Skeletal muscle mitophagy and mitochondrial dynamics in diet and exercise

Several cell culture and transgenic mouse models studies have investigated how skeletal muscle mitophagy and dynamics are influenced by diet and exercise. Following starvation, C2C12 mouse skeletal muscle cells exhibit an elongated mitochondrial network with concomitant increases in the more efficient dimeric and oligomeric ATPase forms, increasing bioenergetic efficiency (Gomes, Benedetto and Scorrano, 2011). The dynamic shift toward a more fused state was the result of suppressed Drp1-mediated fission, which selectively spared mitochondria against cellular autophagy (Gomes, Benedetto and Scorrano, 2011). Conversely, exposure of C2C12 cells to excess palmitate resulted in increased Drp1-mediate fragmentation and insulin resistance, with no changes in Mfn1, Mfn2 or OPA1, and reductions in bioenergetic efficiency, based on greater ROS

and reduced ATP production (Jheng et al., 2012). High-glucose exposure or unsaturated FAs did not stimulate similar adaptations, indicating a saturated FA-specific effect. These findings may be translatable to human skeletal muscle following a study by Montell et al., (2001) which found human primary muscle cells preferentially direct unsaturated fatty acids toward triacylglyceride synthesis, while maintaining insulin sensitivity. Saturated fatty acids meanwhile led to the accumulation of DAGs and reduced insulin-stimulated glucose uptake. Mitochondrial fragmentation was not measured, but given the pro-fission activity of DAGs (Huang et al., 2011), this may link mitochondrial fragmentation to skeletal muscle insulin resistance.

Jheng et al. (2012) provided further support for such a link using diet-induced obese mice fed a high-saturated fat diet, which exhibited mitochondrial fragmentation as well as skeletal muscle insulin resistance. However, pharmacological inhibition of Drp1 reduced mitochondrial fragmentation, improving skeletal muscle insulin signaling and systemic insulin sensitivity. Mitophagy markers were not measured, but mitochondrial content remained stable despite increases in PGC1 α and TFAM, which may suggest a greater rate of mitophagy to compensate for potential increases in mitochondrial biogenesis (Jheng et al., 2012). Increased expression of mtDNA repair enzymes (Jheng et al., 2012) indicates an activation of mitochondrial quality control mechanisms, further supporting an increase in mitophagy. Together, these studies suggest that nutrient deprivation enhances bioenergetic efficiency, promoting a highly fused morphology that is mitophagy resistant. Accordingly, nutrient overload leads to reductions in efficiency, mitochondrial fragmentation and potentially increased rates of mitophagy. In both cases, mitochondrial dynamic shifts were Drp1-dependent, highlighting Drp1 as a potential molecular bridge between mitochondrial bioenergetics and mitophagy. An important regulatory role for Drp1

in metabolic regulation is supported by the finding that its inhibition can prevent insulin-resistance in response to palmitate-induced metabolic stress.

Mitophagy is dependent on autophagosome-mediated degradation, thus autophagy deficiency is synonymous with mitophagy deficiency. Skeletal muscle-specific autophagy deficiency consistently generates dysmorphic, swollen mitochondria with impaired function (Wu et al., 2009; Moresi et al., 2012; Kim et al., 2013). Reductions in mitophagy therefore result in an accumulation of abnormal and dysfunctional mitochondria. Skeletal muscle mitochondria isolated from Atg7 skeletal muscle-specific KO mice had down-regulated OXPHOS genes and reductions in O₂ consumption, cytochrome C activity and ATP content (Kim et al., 2013). Markers of mitochondrial mass, TOM20 and citrate synthase were greater in skeletal muscle of Atg7 KO mice, supporting a reduction in mitophagy. Wu et al., (2009) found comparable mitochondrial defects in a similar Atg7 skeletal muscle-specific KO mouse model. Mitochondria lacked cristae and were less electron dense, but without apparent ETC assembly alterations. Assessment of function found blunted state III and IV respiration, accompanied by significant reductions in activity of complexes I and II. Sub-sarcolemmal (SS) mitochondria were particularly susceptible to developing morphological abnormalities compared to intermyofibrillar mitochondria. This may be significant because SS mitochondria are considered important in signal transduction and substrate transport, and may contribute to the pathogenesis of skeletal muscle insulin resistance and T2D (Ritov et al., 2005). Moresi et al., (2012) used skeletal muscle-specific HDAC1 and 2 KO mice, which exhibit autophagy deficiency akin to Atg7 KO mice. Mitochondria were characterized by an accumulation of NADH and reduction in succinate dehydrogenase, in agreement with the complex I and II activity reported by Wu and colleagues (2009).

Following consumption of a 60% HFD for 13 weeks WT mice developed obesity and insulin resistance, while Atg7 skeletal muscle-specific KO mice were protected (Kim et al., 2013). Despite the abnormal morphology and impaired function of skeletal muscle Atg7 KO mice, insulin-stimulated glucose uptake, glycolysis and glycogen synthesis were increased in response to a HFD. This might be explained by a reduction in mitochondrial OXPHOS activity, leading to a subsequent enhancement in insulin sensitivity to provide a compensatory increase in glycolysis. This may support a role for autophagy/ mitophagy signaling in the development of insulin resistance in response to a HFD. Jheng et al., (2012) used a genetically induced obesity model (ob/ob) to assess mitochondrial morphology and dynamics. Mitochondria isolated from the gastrocnemius were smaller and shorter than lean controls. Assessment of mitochondrial dynamic proteins found significant increases in Drp1 and Fis1, but no notable changes in Mfn1, Mfn2 or OPA1. Inhibition of Drp1 in ob/ob mice improved insulin sensitivity and restored FA oxidation genes, Cpt1b, Lcad, Mcad and Acsl1, which were up-regulated in ob/ob mice, to baseline levels. These findings reiterate the importance of Drp1 and mitochondrial fission in mediating skeletal muscle metabolism.

In addition to changes in skeletal muscle metabolism, obesity is associated with a reduced oxidative phenotype (Boudina et al., 2005; Coen et al., 2013). Lira et al., 2013 investigated the link between oxidative phenotype and mitophagy in mice over-expressing PGC1 α . These mice possess a greater oxidative capacity resulting from a larger mitochondrial population. Basal autophagy flux was increased along with the mitophagy marker, BNIP3, but without increases in autophagy protein expression. The authors postulated that the increased autophagic flux was

representative of increased basal mitophagy in response to increased mitochondrial content and oxidative capacity. Reductions in oxidative capacity as a result of obesity or the development of obesity as a result of reduced oxidative capacity may be linked to altered mitophagy.

It is clear that metabolic disease is associated with changes in mitochondrial function, dynamics and autophagy, which have been related to reductions in mitochondrial quality control. The studies discussed so far in this review support a role for autophagy, mitophagy, mitochondrial dynamics and function in mediating the development and/ or progression of metabolic disease states. Dietary habits, particularly HFDs, and exercise are two frequently studied environmental stimuli associated with inducing and combating metabolic disease, respectively. In light of this review's current findings, further investigation into the interactions between HFDs and exercise, and mitophagy and mitochondrial dynamics is warranted. While cell culture and transgenic rodent models provide important reductionist insights as to how mitophagy and mitochondrial dynamics operate in skeletal muscle they may not represent realistic responses to environmental cues such as HFDs and exercise. In order to assess the current understanding of skeletal muscle mitophagy and mitochondrial dynamics in response to HFDs and exercise a systematic review of the literature was undertaken.

Systematic review

Methods

PubMed, Web of Science and CABDirect were systematically searched for two sets of criteria. The first, related to the influence of metabolic disease and dietary fat on mitophagy in skeletal

muscle, and the second to the effects of exercise on mitophagy in skeletal muscle. Results were compiled, duplicates removed and inclusion/ exclusion criteria applied.

High-fat diet and mitophagy

Variants of three separate terms relating to metabolic disease and diet, mitophagy and mitochondrial dynamics/ function and skeletal muscle were searched in combination. A complete archive of all search terms is shown in table 2. A total of 171 unique search terms were generated, accumulating 513 searches across all three databases.

Table 2. List of search terms for metabolic disease and mitophagy.

Term 1	Term 2	Term 3
Caloric restriction	Apoptosis	Skeletal muscle
Diabetes	Autophagy	
Diet	Cell Death	
Glucose intolerance	Mitophagy	
High-fat diet	Mitochondrial degradation	
High-fat fed	Mitochondrial dynamics	
High-fat feeding	Mitochondrial dysfunction	
High-fat meal	Mitochondrial function	
Insulin resistance	Mitochondrial turnover	
Lipotoxicity		
Macronutrient composition		
Macronutrient intake		
Metabolic disease		
Metabolic flexibility		
Metabolic inflexibility		
Metabolic syndrome		
Obesity		
Overconsumption		
Overweight		

Endurance training and mitophagy

Variants of three separate terms relating to aerobic exercise, mitophagy and mitochondrial dynamics/ function and skeletal muscle were searched in combination. A complete archive of all

search terms is shown in table 3. A total of 45 unique search terms were generated, accumulating 135 searches across all three databases.

Table 3. List of search terms for aerobic exercise and mitophagy.

Term 1	Term 2	Term 3
Aerobic Exercise	Apoptosis	Skeletal muscle
Aerobic Training	Autophagy	
Endurance exercise	Cell Death	
Endurance training	Mitophagy	
Running	Mitochondrial degradation	
	Mitochondrial dynamics	
	Mitochondrial dysfunction	
	Mitochondrial function	
	Mitochondrial turnover	

Inclusion and exclusion criteria

Inclusion criteria included published peer-reviewed original research articles, English language, and measures conducted in skeletal muscle. While human-only studies were preferable, due to the low number, relevant animal models were accepted. Studies investigating skeletal muscle mitophagy with supplements, aging, myopathies, injuries or other diseases were excluded.

Search results

High-fat diet and mitophagy

The databases PubMed, Web of Science and CABDirect were systematically searched. PubMed returned a total of 2428 results, of which 787 were unique and 1641 were duplicates. Web of Science returned 8304 results, of which 2651 were unique and 5653 were duplicates. CABDirect returned 1643 results, of which 428 were unique and 1215 were duplicates. Combined unique results from all three databases produced 3866 hits, of which 2787 were unique and 1079 were duplicates. Following application of inclusion and exclusion filters, and a review of article reference lists, 8 articles that looked specifically at HFDs and mitophagy or mitochondrial dynamics.

Endurance training and mitophagy

The databases PubMed, Web of Science and CABDirect were systematically searched. PubMed returned a total of 239 results, of which 167 were unique and 72 were duplicates. Web of Science returned 599 results, of which 375 were unique and 224 were duplicates. CABDirect returned 553 results, of which 45 were unique and 508 were duplicates. Combined unique results from all three databases produced 587 hits, of which 411 were unique and 176 were duplicates. Following application of inclusion and exclusion filters, and a review of article reference lists, 5 articles were found that looked specifically at exercise and mitophagy or mitochondrial dynamics.

Review of inclusion criteria matched articles

Mitophagy and mitochondrial dynamics in response to a HFD

The 8 qualifying articles investigated mitophagy, dynamics and functions following acute lipid infusions in skeletal muscle of humans as well as following HFDs of varying composition in skeletal muscle of humans, rats and mice. Three human studies qualified, two of which used lipid infusion protocols and vastus lateralis muscle biopsies in non-obese healthy male and female participants (Chavez et al., 2010; Brand et al., 2011). Brand et al., (2011) utilized a 6-hour lipid infusion in tandem with a hyperinsulinemic-euglycemic clamp. Participants experienced significant reductions in insulin sensitivity, but without any changes in intrinsic mitochondrial function or content assessed by oxygen consumption in response to substrate stimulation in permeabilized muscle fibers, and citrate synthase, respectively. In comparison, Chavez et al., (2010) completed a more elaborate and detailed investigation of mitochondrial parameters in response to an 8-hour lipid infusion, which induced acute insulin resistance. Following lipid infusion, ex vivo confocal microscopy found a 33% reduction in mitochondrial membrane potential, correlated with increases in plasma FFAs. Unfortunately, membrane potential-sensitive mitochondrial proteins Drp1, OPA1 and Parkin were not measured. However, mitochondrial content assessed via mtDNA/ nDNA and citrate synthase showed no difference following the infusion. Mitochondrial morphology and ATP content were also unchanged in response to the 8-hour lipid infusion, and SS and IMF mitochondrial responses were matched. Acute lipid infusion, which may reflect an individual high-fat meal, suggests that with the exception of membrane potential, mitochondria are resistant to short term increases in serum FFAs and seemingly unrelated to insulin resistance. Mitochondrial dynamic and mitophagy protein response was not

characterized though, which may provide important information as to whether lipid induced loss of membrane potential is sufficient to trigger dynamic and mitophagy flux.

The qualifying rodent HFD models provide several conflicting results, confounded by the type of rodent and level of fat contained in the respective diets. Hoeks et al., (2008) and van den Broek et al., (2009) used male Wistar rats fed a HFD containing approximately 46% of calories from fat for 8 weeks and 2.5 or 25 weeks, respectively. Following 8 weeks of HFD consumption, HFD and control rats were of similar body mass, but HFD rats were found to have an 8-fold increase in intramyocellular lipid (IMCL) content. Increased HFD-induced IMCL was not associated with changes in mitochondria function (state III and IV respiration or RCR) or ROS production (Hoeks et al., 2008). PGC1 α protein content doubled, but without an accompanying change in TFAM. Citrate synthase was similar between HFD and control rats, but mtDNA copy number indicated a significant decrease in mitochondrial content (Hoeks et al., 2008). This may suggest that mitophagy was highly active, but no markers were measured to confirm, alternatively it could indicate significant increases in non-specific autophagy. However, since citrate synthase did not change, the reduction in mtDNA copy number may reflect a loss of mtDNA rather than mitochondria. While mitochondrial function remained intact, 8 weeks may have been insufficient to induce changes. Wistar rats that consumed a similar HFD diet for 25 weeks showed signs of intrinsic mitochondrial dysfunction and altered mitochondrial turnover (van der Broek et al., 2009). Whole-body insulin sensitivity was reduced in accordance with increased IRS1^{Ser307} phosphorylation in skeletal muscle. Skeletal muscle exhibited significantly increased IMCL and mitochondrial content, assessed through mtDNA copy number, accompanied by a greater PGC1 α content. Skeletal muscle oxidative capacity and OXPHOS enzyme activity increased implying an

adaptation to a 25-week HFD. In vitro analysis of mitochondrial function found no dysfunction, which conflicted with in vivo assessments that demonstrated HFD fed rats required 42% more mitochondria to maintain oxidative capacity compared to controls. This suggests a reduction in bioenergetic efficiency, which might be expected to increase mitophagy to maintain mitochondrial quality. However, a series of studies discussed earlier (Jheng et al., 2012; Gomes, Benedetto and Scorrano, 2011; Melser et al., 2013; MacVicar and Lane, 2014) that examined the link between bioenergetic efficiency and mitophagy markers suggested that bioenergetic efficiency may negatively regulate mitophagy. It has been suggested that bioenergetic efficiency might become reduced to allow an increased rate of FA metabolism as a compensatory mechanism for increased IMCL following a HFD to protect against oxidative stress (Costford et al., 2008; Liesa and Shirihai, 2013). Under such circumstances it may be beneficial to down-regulate mitophagy to maintain a less efficiency, but large mitochondrial population. van den Broek et al., (2009) noted increased expression of UCP3 following 25 weeks of HFD, which supports an attempt to increase uncoupling and therefore minimize efficiency.

Two further studies used C57BL mice fed a 60% HFD for 16 weeks (Jheng et al., 2012; Yuzefovych et al., 2013), and a third fed C57BL mice a HF (36%)/ high-sucrose (HS) diet (17.5%) also for 16 weeks (Bonnard et al., 2008). Jheng et al., (2012) did not assess markers of mitophagy, but did find increases in Drp1 and Fis1 in response to a HFD. Mitochondria were also smaller and shorter supporting an increased fission-mediated fragmentation of the network. Mfn1, Mfn2 and OPA1 were unchanged in response to a 16 week HFD. Yuzefovych et al., (2013) found that a 16-week HFD induced mitochondrial dysfunction and mtDNA damage, possibly due to increased oxidative stress. Mitophagy may have been more active following a 16 week 60% HFD because

mtDNA copy number was reduced. However, no supporting measure was provided so the change may have been the result of mtDNA loss rather than organelle degradation. Reductions in TFAM, PGC1 α , MnSOD and mtDNA replication enzymes suggest mitochondrial quality control was impaired following a HFD. Findings by Bonnard and colleagues (2008) imply that a HF/HS diet impairs mitochondrial quality control. Reductions in mtDNA/nDNA, in tandem with citrate synthase reductions suggest that mitochondrial content was reduced following a 16-week HF/HS diet. PGC1 α expression and content were lower following the diet, as were SS and IMF oxidative fibers. mtDNA repair enzymes, POLG2 and SSBP1 were also reduced, supporting the possibility that mitochondrial quality was impaired.

Finally, Samocha-Bonet et al., (2012) investigated the effects of 28 days HF (46%) overconsumption in non-obese sedentary humans. Forty men and women consumed approximately 1040kcal in addition to basal metabolic requirements. Participants experienced peripheral insulin resistance and increased levels of protein carbonyls and urinary F2-isoprostanes, indicators of oxidative stress. Mitochondrial biogenesis marker, PGC1 α initially increased, along with complex I, II and V after 3 days of HF overconsumption. However, levels returns to normal following the full 28 days. No change in citrate synthase or ex vivo CO₂ production was found, indicating that mitochondrial content and function remained stable, although further analysis is required to confirm.

Together, these studies provide an interesting, albeit limited analysis of how mitochondrial dynamics and mitophagy respond to obesogenic HFDs. Unfortunately, all of the findings regarding mitophagy were supposed on the basis of changes in markers of mitochondrial content and

biogenesis, since not a single study measured mitophagy markers such as BNIP3, PINK1 or Parkin. Mitochondrial dynamics were also under studied, which would otherwise provide supporting data to that of mitophagy and lead to better understanding about how mitochondrial quality control is regulated in response to a HFD.

Mitophagy and mitochondrial dynamics in response to exercise

The 6 qualifying articles examined the relationship between oxidative capacity and mitophagy, and the mitophagic response to acute and chronic exercise training in skeletal muscle of mice. The systematic review also produced two articles investigating markers of skeletal muscle mitophagy and dynamics in response to ultra-endurance exercise in humans. Lira et al., (2013) investigated the effects of oxidative capacity on mitophagy and biogenesis using muscle groups of different fiber type composition from mice. Vastus lateralis, plantaris and soleus were used as muscle groups representative of predominantly type II, mixed fiber (type I/II) and type I, respectively. The mitophagy marker BNIP3 was lowest in the more glycolytic vastus lateralis and most abundant in the highly oxidative soleus muscle, while plantaris muscle possessed an intermediate BNIP3 expression. The same pattern was repeated for mitochondrial biogenesis markers, PGC1 α , cytochrome C and Cox4, with the greatest expression in the oxidative soleus muscle group. Measurements were taken in the non-stimulated basal state implying that basal mitophagy is positively correlated with muscle oxidative capacity. Autophagy machinery was also sequentially upgraded in muscle groups of increasing oxidative capacity, supporting oxidative capacity-dependent enhancements in basal mitophagy. Mitochondrial biogenesis markers rose in tandem with increasing markers of mitophagy suggesting that mitochondrial turnover was also most active in mitochondria-rich oxidative tissues. To further test the relationship between mitophagy and

oxidative capacity, Lira et al., (2013) measured the identical markers following 4 weeks of voluntary wheel running in mice. In response to exercise training the mixed fiber plantaris muscle groups developed a more oxidative phenotype. Basal autophagic flux was increased, as was BNIP3, PGC1 α , cytochrome c and Cox4 expression. Examination of oxidative soleus muscle following the same exercise protocol did not elicit further increases in oxidative capacity and accordingly showed no changes in autophagic flux, BNIP3 or biogenesis markers (Lira et al., 2013). Increases in oxidative capacity appear therefore to stimulate higher mitophagy and mitochondrial turnover activity. Although the possibility remains that mitophagy and mitochondrial turnover activity may govern exercise-induced increases in oxidative capacity. He et al., (2012) found that intact autophagy is a pre-requisite for adaptation to exercise, suggesting that mitophagy and mitochondrial turnover might be the adaptive mediator.

These findings raise questions as to how mitophagy is regulated in response to acute exercise. He et al., (2012) found no changes in mitochondrial content or function following 80 minutes of moderate intensity exercise in mice. No individual measures of mitophagy or biogenesis were made so it is not possible to determine whether the stable mitochondrial content was due to a failure to stimulate either mitophagy or biogenesis, or because both were equally up or down-regulated. Grumati et al., (2011) found autophagic puncta merging with mitochondrial networks immediately following 1 hour of graded exercise, possibly indicating exercise-induced mitophagy. Both studies were limited, however, Jamart and colleagues (2013) completed a more comprehensive study. Mice exercised for 90 minutes at a low intensity in both a fed and fasting state. Drp1 mRNA expression was unresponsive to exercise in both a fed and fasting state, but was blunted during fasting exercise. Fission active phospho-Drp1⁶¹⁶ was significantly increased in both

fed and fasted state exercise implying a greater degree of mitochondrial fission. BNIP3 mRNA expression increased with during fasting state exercise, but BNIP3 protein content was significantly reduced following exercise. Increased BNIP3 mRNA demonstrates a stimulation of mitophagy, while reduced protein content suggest BNIP3 degradation and this active mitophagy, in accordance with increased Drp1-mediated fission activity. Parkin protein content was also significantly reduced following fasting state exercise, likely reflecting increased mitophagy in light of Drp1 and BNIP3 results. Importantly, elevated LC3II:LC3I and reduced p62 demonstrate active autophagy, necessary for increased mitophagy. Expression of mitochondrial fusion proteins, Mfn1 and Mfn2 mRNA, were not significantly changed in response to fed or fasting state exercise supporting Drp1 as the master switch controlling mitochondrial dynamic balance in response to exercise. PGC1 α mRNA was significantly increased in response to exercise, independent of fed or fasting state. This may suggest that exercise in a fed state prioritizes biogenesis to increase mitochondrial mass, while fasting state exercise promotes both biogenesis and mitophagy, increasing turnover and thus activating mitochondrial quality control mechanisms. The cause of increased mitophagy is not clear because oxidative stress was not significantly increased in fasting state exercise according to protein carbonyls. While it is possible that mitophagy was increased to support cellular energy demands through the provision of substrates, this seems counterintuitive under fasting state exercise when a high oxidative capacity would be advantageous to meet energy demands through FA-oxidation. Alternatively, BNIP3 and Parkin may have been degraded through an alternative pathway and increases in autophagy may have been non-specific to provide non-mitochondrial energy substrates. The reason for the noted shift toward mitochondrial fission is not clear in the absence of mitophagy.

Research into the relationship between exercise and mitophagy in humans is extremely limited. The systematic review found two human studies that matched the inclusion criteria (Jamart et al., 2012a; Jamart et al., 2012b). The studies were primarily concerned with investigating autophagy (*see* “Human skeletal muscle autophagy and exercise”), and thus provided relatively little information on mitophagy and mitochondrial dynamics. Both studies examined the influence of ultra-endurance exercise in highly endurance trained male participants. Jamart et al., (2012a) analyzed BNIP3, phospho-Drp1, Mfn1, PINK1 and Parkin content in vastus lateralis biopsies taken immediately upon cessation of a 24-hour treadmill run. BNIP3 was unchanged suggesting inactive basal mitophagy. Markers of stress-induced mitophagy, PINK1 and Parkin were assessed, but did not change. In accordance with previous findings (Jamart et al., 2013), phospho-Drp1^{Ser616} was significantly increased and Mfn1 remained unchanged, indicative of a shift toward mitochondrial fission. Without additional measures it is difficult to interpret whether BNIP3, PINK1 and Parkin protein content is the result of inactive mitophagy at the end of 24 hours treadmill running or whether active mitophagy was degrading the markers. However, similar to Jamart et al., (2013) it might be expected that proteins levels would decrease if mitophagy were active. Jamart et al., (2012b) analyzed muscle biopsied from the vastus lateralis of 8 highly endurance trained male runners before and 3 hours after a 200 km race lasting an average of 28 hours. Sample analysis was limited to mRNA and only looked at the basal mitophagy markers BNIP3 and Nix. Both were significantly increased along with markers of increased autophagy flux. Jamart et al., (2013) previously demonstrated that increased BNIP3 mRNA was likely associated with increased mitophagy. If so, the difference between Jamart et al., (2012a) and Jamart et al., (2012b) may be due to the post-run biopsy collection time. Based on the limited available literature, mitophagy, mitochondrial fission and turnover appears to be positively correlated to

muscle oxidative capacity and increases in response to exercise training that induces adaptive increases in muscle oxidative capacity. Acute exercise bouts of moderate and ultra-endurance length likely increase mitophagy and promote mitochondrial fission, but require further more detailed studies.

Concluding remarks and future research

Autophagy, mitophagy and mitochondrial dynamics represent constitutive, but under studied components of mitochondrial quality control. Further, given the importance of skeletal muscle in systemic metabolic regulation it is an area that has been neglected. The processes that govern their interactions remain unclear, particularly in light of the complex web of kinases and transcription factors that respond in a stimuli-specific manner dependent upon, in the case of skeletal muscle, the oxidative characteristics of the surrounding myofibers. Currently, much of our understanding rests on a small group of rodent studies using a variety of HFD or exercise protocols. As such there is minimal clinically relevant literature underpinning how mitophagy and mitochondrial dynamics interact with HFDs and exercise, increasing the challenge of understanding how mitophagy and dynamics are implicated in metabolic disease. Future studies should aim to use current knowledge of mitophagy markers and mediators of mitochondrial dynamics to characterize how these pathways respond to HFDs, exercise or different oxidative capacities of within human skeletal muscle.

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CHAPTER III

Specific Aims

THE SPECIFIC AIMS OF THIS DISSERTATION ARE:

SPECIFIC AIM 1: To determine the influence of a single high-fat, high-saturated fat meal on skeletal muscle autophagy and mitophagy in healthy, non-obese sedentary individuals.

Hypothesis: We hypothesize that a single high-fat, high-saturated fat meal will suppress skeletal muscle autophagy and mitophagy in healthy, non-obese sedentary individuals.

Objective: Measure markers of autophagy and mitophagy in skeletal muscle biopsy samples, obtained from healthy, non-obese sedentary males, taken prior to, and 4 hours after a high-fat, high-saturated fat meal.

SPECIFIC AIMS 2: To determine the influence of a short-term high-fat, high-saturated fat diet on skeletal muscle autophagy and mitophagy in healthy, non-obese sedentary individuals.

Hypothesis 1: We hypothesize that a short-term, high-fat, high-saturated fat diet will reduce basal skeletal muscle autophagy and mitophagy in healthy, non-obese sedentary individuals.

Hypothesis 2: We hypothesize that a short term high-fat, high-saturated fat diet will alter the skeletal muscle autophagy and mitophagy response to a single high-fat, high-saturated fat meal in healthy, non-obese sedentary individuals.

Objective 1: Measure markers of autophagy and mitophagy following a 12 hour overnight fast, in skeletal muscle biopsy samples, obtained from healthy, non-obese sedentary males, prior to and after a 5-day high-fat, high-saturated fat diet.

Objective 2: Compare changes to markers of skeletal muscle autophagy and mitophagy in response to a single high-fat, high-saturated fat meal before and after a 5-day high-fat, high-saturated fat diet in healthy, non-obese sedentary males.

SPECIFIC AIM 3: To compare markers of skeletal muscle autophagy and mitophagy between healthy, non-obese sedentary individuals and endurance-trained runners in response to a single high fat, high-saturated fat meal.

Hypothesis 1: We hypothesize that endurance-trained runners will exhibit elevated basal skeletal muscle autophagy and mitophagy compared to healthy, non-obese sedentary individuals.

Hypothesis 2: We hypothesize that endurance-trained runners will exhibit attenuated skeletal muscle autophagy and mitophagy responses to a single high-fat meal challenge, compared to healthy, non-obese sedentary individuals.

Objective 1: Measure and compare markers of autophagy and mitophagy, following a 12 hour overnight fast, in skeletal muscle biopsy samples obtained from healthy, non-obese sedentary males and endurance-trained male runners.

Objective 2: Compare changes to markers of autophagy and mitophagy in skeletal muscle of healthy, non-obese sedentary males and endurance-trained male runners before and after a single high-fat, high saturated fat meal.

SPECIFIC AIM 4: To compare skeletal muscle metabolic flexibility in healthy, non-obese sedentary individuals and endurance-trained runners following a single high fat, high-saturated fat meal.

Hypothesis 1: We hypothesize that endurance-trained male runners will exhibit greater skeletal muscle metabolic flexibility compared to healthy, non-obese sedentary males, and this will be associated with elevated autophagy and mitophagy.

Hypothesis 2: We hypothesize that greater skeletal muscle metabolic flexibility in endurance-trained runners, if observed, will be associated with elevated markers of autophagy and mitophagy.

Objective: Measure and analyze correlations between metabolic flexibility and markers of autophagy and mitophagy in skeletal muscle of healthy, non-obese sedentary males and endurance-trained male runners.

CHAPTER IV

The effect of a 5-day high-fat diet on skeletal muscle autophagy and mitophagy

ABSTRACT

Habitual high-fat intake is associated with reduced skeletal muscle insulin sensitivity, which often precedes the development of type II diabetes and related comorbidities. Insulin resistance and type II diabetic patients' often display impaired mitochondrial function, which may indicate a loss of mitochondrial quality control. Regulation of mitochondrial quality is dependent on autophagy and mitophagy for the removal of dysfunctional mitochondria. High-fat diets have also been linked to impaired skeletal muscle mitochondrial function, insulin resistance, and suppression of autophagy and mitophagy. To date, the effects of dietary fat intake on human skeletal muscle autophagy and mitophagy have not been investigated. Thirteen healthy, sedentary, non-obese college age males volunteered for the study to investigate the effects of high-fat feeding on markers of skeletal muscle autophagy and mitophagy. Participants' consumed a two-week controlled diet prior to beginning the high-fat diet. Following the controlled feeding period, participants' completed a high-fat meal challenge, which took a biopsy from the vastus lateralis after a 12h overnight fast and again 4h after a high-fat meal. Participants' then consumed a high-fat diet for 5-days before repeating the high-fat meal challenge. Skeletal muscle demonstrated signs of reduced autophagy in response to a high-fat diet and an altered autophagy response to a high-fat meal following the 5-day high-fat diet. Mitophagy markers suggested reduced activity following a high-fat diet and in response to a high-meal, although evidence was contradictory. These findings may suggest that a short-term high-fat diet reduced skeletal muscle autophagy. Reductions in autophagy following a high-fat diet, combined with reduced expression of mitophagy proteins may indicate impaired

mitophagy in response to a high-fat diet. Data on mitophagy markers were inconclusive though, and a reduction in autophagy activity could not be confirmed.

INTRODUCTION

Obesity represents a global epidemic with extensive health and economic implications. Reduced insulin sensitivity in skeletal muscle is a key characteristic of obesity that typically appears prior to the onset of type II diabetes mellitus (T2D) (Lillioja et al., 1988; Warram et al., 1990). Controversy still remains as to the exact mechanism of skeletal muscle insulin resistance, but an inequality between the production and removal of lipid metabolites leading to an accretion of intramyocellular lipids (IMCL) is a commonly cited factor (Goodpaster et al., 2001; Itani et al., 2002; Perseghin et al., 2002). Skeletal muscle from obese, insulin resistant, and T2D patients' is further characterized by an accumulation of morphologically and functionally impaired mitochondria, defined by reductions in oxidative capacity and increased reactive oxygen species (ROS) production (Kelley et al., 2002; Mootha et al., 2003; Ritov et al., 2010; Wijngaarden et al., 2013). Mitochondria are central to energy metabolism, regulating basal substrate selection (Gottlieb and Carreira, 2010; van de Weijer et al., 2013), a role emphasized in skeletal muscle, which is responsible for a majority of whole-body glucose disposal and fatty acid oxidation (Baron et al., 1988; van Hall et al., 2002). Conjecture remains as to the causal relationship between IMCL accumulation and mitochondrial dysfunction, although the deleterious effects of excess lipid intake on skeletal muscle insulin sensitivity may be mediated via increased mitochondrial ROS production (Anderson et al., 2009)

Excess lipid intake has also been associated with a suppression of autophagy, compromised mitochondrial quality control, and the development of insulin resistance in the skeletal muscle of rodents (Bonnard et al., 2008; van der Broek et al., 2009; Komatsu and Ichimura, 2010; He et al., 2012; Jheng et al., 2012; Yuzefovych et al., 2013). Autophagy describes lysosome-dependent protein degradation, including the removal of dysfunctional mitochondria via the organelle-specific process of mitophagy (Rikka et al., 2011; Lemasters, 2005). High-fat diet-induced autophagy suppression, leading to reductions in mitophagy and the eventual loss of mitochondrial quality control may represent a new investigative avenue into the etiology of skeletal muscle insulin resistance. It is not currently clear if the suppressive effects of high-fat feeding on autophagy in rodents are translatable to human skeletal muscle, or whether mitophagy markers are similarly diminished by excess fat intake, and if so, what severity of high-fat feeding is required to alter these pathways. The purpose of the current study was to investigate the impact of a high-fat meal and a high-fat diet on markers of autophagy and mitophagy in skeletal muscle of healthy, sedentary humans.

MATERIALS AND METHODS.

Participants’. Thirteen healthy, non-obese, sedentary (≤ 2 days, 20 min/day of low-intensity physical activity) males, aged 18-40 served as participants’ for the study. Participants’ were weight stable ($< \pm 2.5$ kg) for the past 6 months with a BMI > 18 or < 30 kg/m² and were not under the influence of any medications known to affect study measures. All participants’ had blood pressure $< 140/90$ mmHg, fasting glucose < 100 mg/dL, LDL cholesterol < 130 mg/dL, total cholesterol < 200 mg/dL, triglycerides < 250 mg/dL, and percentage of habitual calorie intake composed of $< 40\%$ fat and 15% saturated fat. Participants’ were non-smokers with no family history of disease. All study procedures were approved by the Virginia Polytechnic Institute and State University

Institutional Review Board. Prior to participation all procedures, benefits and any potential risks associated with the study were explained to participants' before written consent was provided.

Experimental design. Following successful completion of screening procedures, participants' underwent a two week isocaloric controlled feeding period, followed by a 5 day isocaloric high-fat, high-saturated fat diet (HFD) (see figure 1). Participants' completed a high-fat meal (HFM) challenge (see figure 2), before and after the 5 day HFD. Muscle biopsies were taken immediately prior to, and 4 hours after a HFM for assessment of skeletal muscle autophagy and mitophagy, and metabolic response and adaptation.

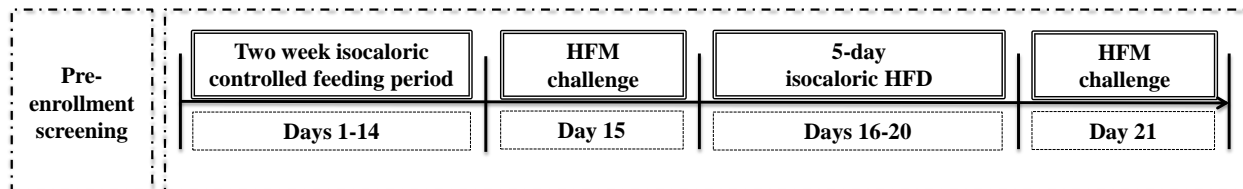


Figure 1. Schematic of study design. Participants' completed a pre-enrollment screening prior to consuming an isocaloric diet for two weeks. Following which, participants' completed a HFM challenge (figure 2) before starting a 5 day HFD. A HFM challenge was repeated post-HFD. HFM, high-fat meal; HFD, high-fat, high-saturated fat diet.

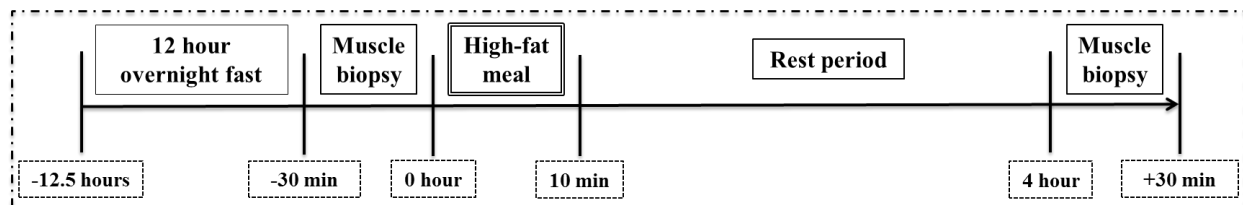


Figure 2. Schematic of HFM challenge design. Participants' fasted for 12 hours overnight prior to a baseline skeletal muscle biopsy. Participants' consumed a HFM and rested for 4 hours before completing a second follow-up skeletal muscle biopsy. HFM, high-fat meal.

Diet Standardization and Food Delivery Procedures. Both the standardized diet and HFD operated on a 7 day cycle of menus consisting of meals and snacks with two optional snack modules (\pm 250 kcals). Diets were planned using Nutrition Data System for Research (NDSR) software version 2012 (University of Minnesota) by a registered dietitian. The two week isocaloric controlled feeding period required participants' to consume planned meals consisting of approximately 55% carbohydrate, 30% fat (< 10% saturated fat), and 15% protein. The 5-day isocaloric HFD was composed of planned meals and contained approximately 55% fat (45% saturated fat equal to 25% of total calories) 30% carbohydrate, and 15% protein. Diets aimed to provide 3 g of fiber per 500 kcal (\pm 5 g). All meals were prepared in the Department of Human, Nutrition, Foods and Exercise metabolic kitchen. Participants' ate breakfast in the Laboratory for Eating Behavior and Weight Management every day and took the remaining food for the day with them. Daily energy needs were determined using the Harris-Benedict equation (Institutes of Medicine). Four-day food records were used to confirm that habitual diets contained less than 40% of total calories from fat. Participants' were weighed in each day at the lab prior to breakfast. A trend of > 1.0 kg weight loss or gain was offset by adding or subtracting 250 kcal food modules with the same macronutrient composition as the overall diet. All uneaten items were returned to the metabolic kitchen for weighing. Participants' were not be permitted to consume any caffeine or alcohol for the duration of the study.

HFM challenge. Participants' arrived at the laboratory following a 12 hour overnight fast. Biopsies of the vastus lateralis muscle were taken before and 4 hours after a HFM. A HFM consisted of two Jimmy Dean sausage, egg and cheese biscuits containing 768 kcal, composed of 53.9 g fat (25.5 g saturated fat), 44.2 g carbohydrate and 25.7 g protein. Participants' were required to consume the HFM within 10 minutes and remain seated and awake for the duration of the meal challenge. Following the initial biopsy participants' were fitted with an intravenous catheter in the antecubital vein for baseline and hourly blood sampling. Pre and post biopsies were taken from separate legs.

Measurements.

Body mass and composition. Body weight was measured to the nearest ± 0.1 kg on a digital scale (Model 5002, Scale-Tronix, White Plains, NY). Height was measured to the nearest ± 0.1 cm using a stadiometer. Body composition (total fat and fat-free mass) was analyzed by dual-energy x-ray absorptiometry (General Electric, Lunar Digital Prodigy Advance, software version 8.10e Madison, WI).

Muscle biopsies. Biopsies were taken from the vastus lateralis muscle using a suction-modified Bergstrom needle technique (Bergstrom, 1962). An area of skin in the region of the vastus lateralis was shaven and cleansed with a povidine-iodine solution. The skin, adipose tissue and skeletal muscle fascia was anesthetized using 10mL lidocaine (1%). The skin was incised (0.75 cm) with a #10 scalpel, and the fascia fibers were separated with the blunt edge of the scalpel. The Bergstrom needle (5 mm) was inserted into the vastus lateralis and suction applied. Muscle used to assess pyruvate dehydrogenase (PDH) activity and metabolic flexibility was immediately placed in SET buffer (0.25 M Sucrose, 1 mM EDTA, 0.01 M Tris-HCl and 2 mM ATP) and stored on ice until homogenization (~25 min). Muscle tissue used for western blotting was placed in ice-cold cell

lysis buffer (50 mM Tris-HCl, EDTA 1 mM, NaCl 150 mM, SDS 0.1%, sodium deoxycholate 0.5%, Igepal Ca 630 1%, pH 7.5) with halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Pittsburg, PA), and 0.1 mM bafilomycin A (Invivogen, San Diego, CA), then snap-frozen in liquid nitrogen. Protein samples were stored at -80°C for later analysis.

Muscle homogenization and western blot analysis. Frozen muscle tissue samples were homogenized in ice-cold lysis buffer containing 0.1mM bafilomycin A1 in a Bullet Blender Homogenizer (Next Advance, NY) using 1.0mm Zirconium Oxide beads (Next Advance). Samples were further homogenized with a 23-gauge needle, and centrifuged at 14,000 g for 15 min at 4°C to remove insoluble components. Supernatant protein concentrations were determined spectrophotometrically using the bicinchoninic acid assay (BCA) (Thermo Scientific). Lysis buffer was added to samples for adjustment to equal concentrations and combined with equal volumes 2 x Laemmli buffer and heated for 5 min at 95°C. Equal amounts of protein were separated on SDS-PAGE gels (Criterion TGX Stain-Free Gels, Bio-Rad, Hercules, CA), which were activated via ultra violet light exposure (ChemiDoc Touch Imaging System, Bio-Rad) prior to transfer. Proteins were transferred to PVDF membranes using a Trans-Blot Turbo Transfer System (Bio-Rad), which were then imaged (Bio-Rad) for quantification of total lane protein. PVDF membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk or 5% bovine serum albumin prior to overnight incubation at 4°C with primary antibodies. Membranes were probed with primary antibodies against FoxO3a (cat# 17026), LAMP2 (cat# 25632), MFN1 (cat# 57602), MFN2 (cat# 56889), Parkin (cat# 15954), PINK1 (cat# 23707) (Abcam, Cambridge, MA), Beclin-1 (cat# 3738), Bcl-2 (cat# 2870s), LC3B (cat# 2775) (Cell Signaling, Danvers, MA), total OXPHOS cocktail (cat# MS601) (MitoSciences, Eugene, OR), DRP1 (cat# 110-55288) (Novus Biologicals, Littleton, CO), SQSMT1/ p62 (cat# 28359) (Santa Cruz Biotechnology, Dallas, TX),

ULK1 (cat# A7481) (Sigma-Aldrich, St Louis, MO) and phospho-specific primary antibodies against FoxO3a (Thr³², cat# 26649), Parkin (Ser⁶⁵, cat# 154995) (Abcam), DRP1 (Ser⁶¹⁶, cat# 3455), ULK1 (Ser⁵⁵⁵, cat# 5869 – equivalent to human Ser⁵⁵⁶) (Cell Signaling), and PINK1 (Thr²⁵⁷, cat# 68-0057-100) (Ubiquigent, Dundee, Scotland, UK). Following primary antibody incubation membranes were incubated for 1 hour at room temperature with HRP-conjugated anti-mouse, anti-rabbit, anti-sheep (Jackson Immuno Research Laboratories, West Grove, PA) and anti-goat (Santa Cruz) secondary antibodies. Proteins were visualized via chemiluminescence (Clarity Western ECL Substrate, Bio-Rad, or SuperSignal West Femto, Thermo Scientific) and quantified using Image Lab Software (v5.2.1, BioRad) and normalized to total lane protein content. Molecular weight was determined by Precision Plus Protein Unstained Standards (Bio-Rad).

Statistics. Two-way repeated measures analysis of variance was used to determine differences in meal responses pre and post-HFD. Multiple comparisons were performed using a Tukey post-hoc analysis. Independent t-tests were used to compare percent change in protein levels between pre and post-meal time points, before and after a HFD. Correlations were examined via multivariate analysis. Data that did not follow a normal distribution were log base 10, or square root transformed. All data are expressed as means \pm standard error of the mean (SEM). The significance level was set *a priori* at $\alpha = .05$.

RESULTS

Participant characteristics. Participant characteristics are displayed in table 1. There were no significant changes in body mass, BMI, lean mass, fat mass or body fat percentage following either the two week controlled feeding period or the 5 day HFD.

Table 1. Body composition.

Characteristics	Baseline	Pre-HFD	Post-HFD
Age (yrs)	22.2 ± 0.4	-	-
Body mass (kg)	72.55 ± 2.9	72.1 ± 3.2	71.9 ± 2.9
BMI	22.9 ± 0.8	23.1 ± 0.9	23.0 ± 0.8
Lean mass (kg)	53.1 ± 1.5	53.2 ± 1.6	54.3 ± 1.7
Fat mass (kg)	14.1 ± 1.4	15.8 ± 1.8	15.6 ± 1.9
Percent body fat	21.0 ± 1.3	22.0 ± 1.7	21.4 ± 1.6

Values are mean ± SEM.

Autophagy markers. The ratio of LC3II to LC3I was significantly reduced in response to a HFM overall, although no significant differences were found for either of the HFMs individually (figure 1). The fasting state LC3 ratio was not significantly altered followed a 5 day HFD, although the percent change in the ratio of LC3II to LC3I in response to a HFM was reduced after consumption of a 5 day HFD, representing a strong trend ($p = 0.051$). The post-HFM reduction in skeletal muscle autophagy implied by these data was not supported by significant changes in SQSMT1/ p62 and total LC3II. Neither, SQSMT1/ p62 or total LC3II were affected during fasting or in response to a HFM following consumption of a 5 day HFD (figure 3). Levels of total LC3 (sum of LC3II and LC3I) were not significantly influence by a HFM prior to, or post HFD. However, a significant diet affect was found, indicating greater total LC3 following a HFD independent of a HFM. No significant differences between pre and post-HFM time points before and after a 5-day HFD were found. The lysosome membrane protein, LAMP2, demonstrated no significant change in response to a HFM, or during fasting following a HFD. The LAMP2 response to a HFM was similarly unaltered by a 5 day HFD, indicating no changes in skeletal muscle lysosome content (figure 3).

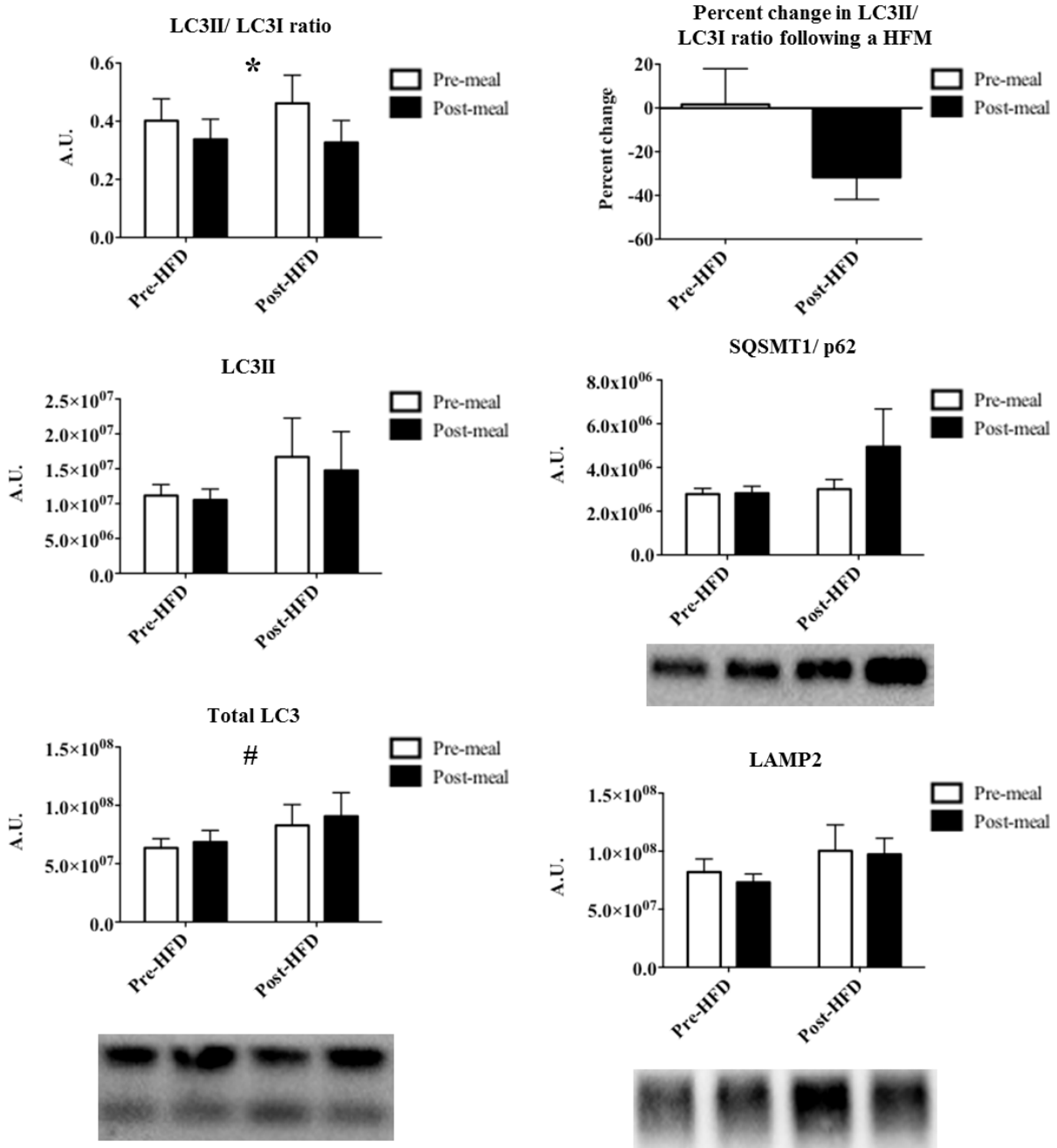


Figure 3. Assessment of markers of skeletal muscle autophagy activity. Differences in protein expression of key autophagy activity markers in response to a HFM, before and after a 5 day HFD.

Representative western blots are included below their respective graphs. Data are presented as mean \pm SEM. *, significant HFM effect; #, significant HFD effect.

To determine whether high-fat feeding affected regulation of autophagy pathways expression of the anti-autophagy regulator, Bcl-2, and pro-autophagy, Beclin-1, were analyzed. Total Bcl-2 content did not respond significantly to a HFM before or after a HFD, and was not significantly affected during fasting following a 5 day HFD. Expression of total Beclin-1 did increase significantly overall following a 5 day HFD, but was not significantly altered between pre and post-HFM time points before or after a HFD. Total Beclin-1 was not significantly affected by a HFM or during fasting state metabolism after consumption of a HFD. Upstream regulators of autophagy, total ULK1 and FoxO3a were also analyzed, in addition to their phosphorylation-dependent active forms, ULK1^{Ser556} and FoxO3a^{Thr32}. No significant differences were found for total and phosphorylated ULK1 or total and phosphorylated FoxO3a in response to a HFM, during fasting following a HFD, or in response to a HFM after a 5 day HFD. Comparison of the relationship between total and phosphorylated ULK1, and total and phosphorylated FoxO3a revealed no significant alteration in protein activity followed a HFM, either before or after a HFD (figure 4).

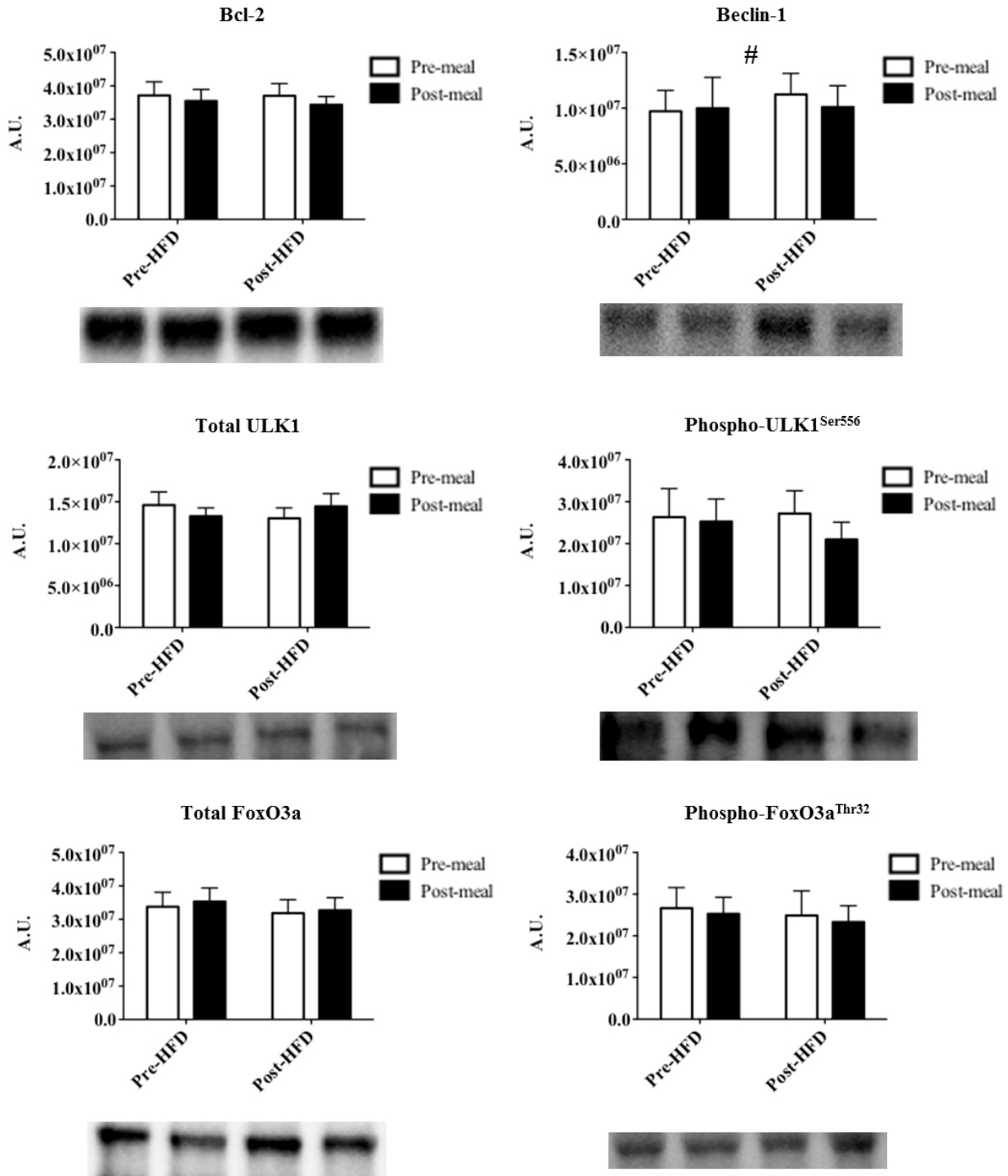


Figure 4. Assessment of autophagy regulator protein content. Differences in protein expression of autophagy pathway regulators in response to a HFM, before and after a HFD. Representative

western blots are included below their respective graphs. Data are presented as mean \pm SEM. #, significant HFD effect.

Mitophagy markers. Analysis of total PINK1 found no significant meal response, or difference in fasting PINK1 levels post-HFD. The percent change in total PINK1 following a HFM was increased post-HFD, suggesting the presence of a HFD-dependent effect on the HFM response. The difference was not significant, but did represent a trend ($p = 0.073$) (figure 5). Changes in the activity of PINK1 were assessed via measurement of active phospho-PINK1^{Thr257}. Levels of phospho-PINK1^{Thr257} were not significantly influenced following a HFM before or after a HFD, or during fasting state metabolism in response to a HFD. Comparison of the relationship between total and phosphorylated PINK1 in response to a HFM before and after a HFD found a strong trend toward a diet and phosphorylated PINK1 interaction ($p = 0.054$) (figure 5). These data may support a greater ratio of total to phosphorylated PINK1, indicative of reduced mitophagy in response to a HFM following a 5 day HFD, although data were not significant.

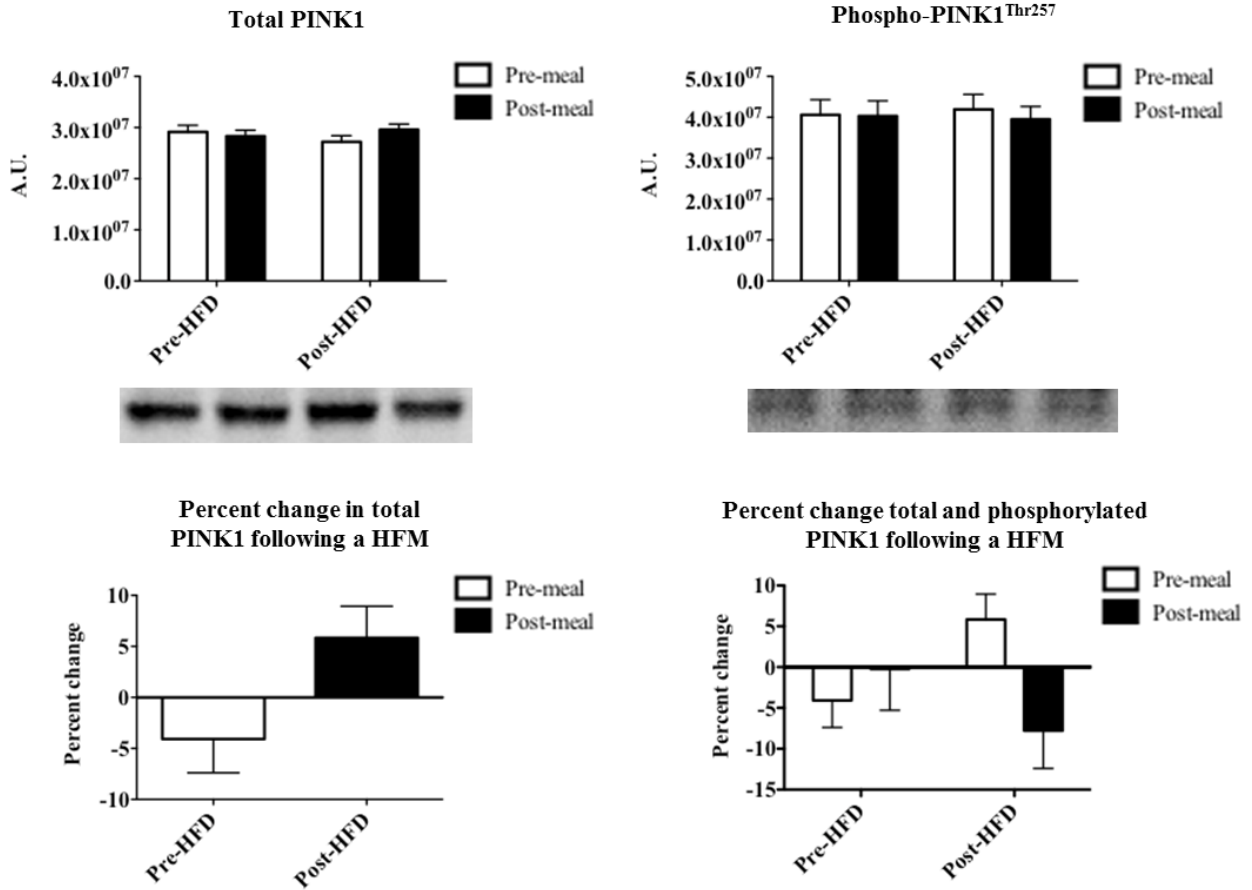


Figure 5. Assessment of changes in skeletal muscle protein content of mitophagy markers.

Differences in protein expression of PINK1, a key regulator and initiator of mitophagy in response to a HFM, before and after a HFD. Representative western blots are included below their respective graphs. Data are presented as mean \pm SEM.

In addition to PINK1, total Parkin and its active phosphorylated form, phospho-Parkin^{Ser65}, were analyzed to assess mitophagy. Levels of total and phosphorylated Parkin were unaltered by a HFM or a HFD (figure 6). Percent change in total Parkin following a HFM was significantly greater than the percent change in phospho-Parkin^{Ser65} following a HFM, independent of a HFD, thus indicating

a reduction in the phosphorylation status and activity of Parkin (figure 6). These data may suggest that mitophagy activity is reduced following a HFM.

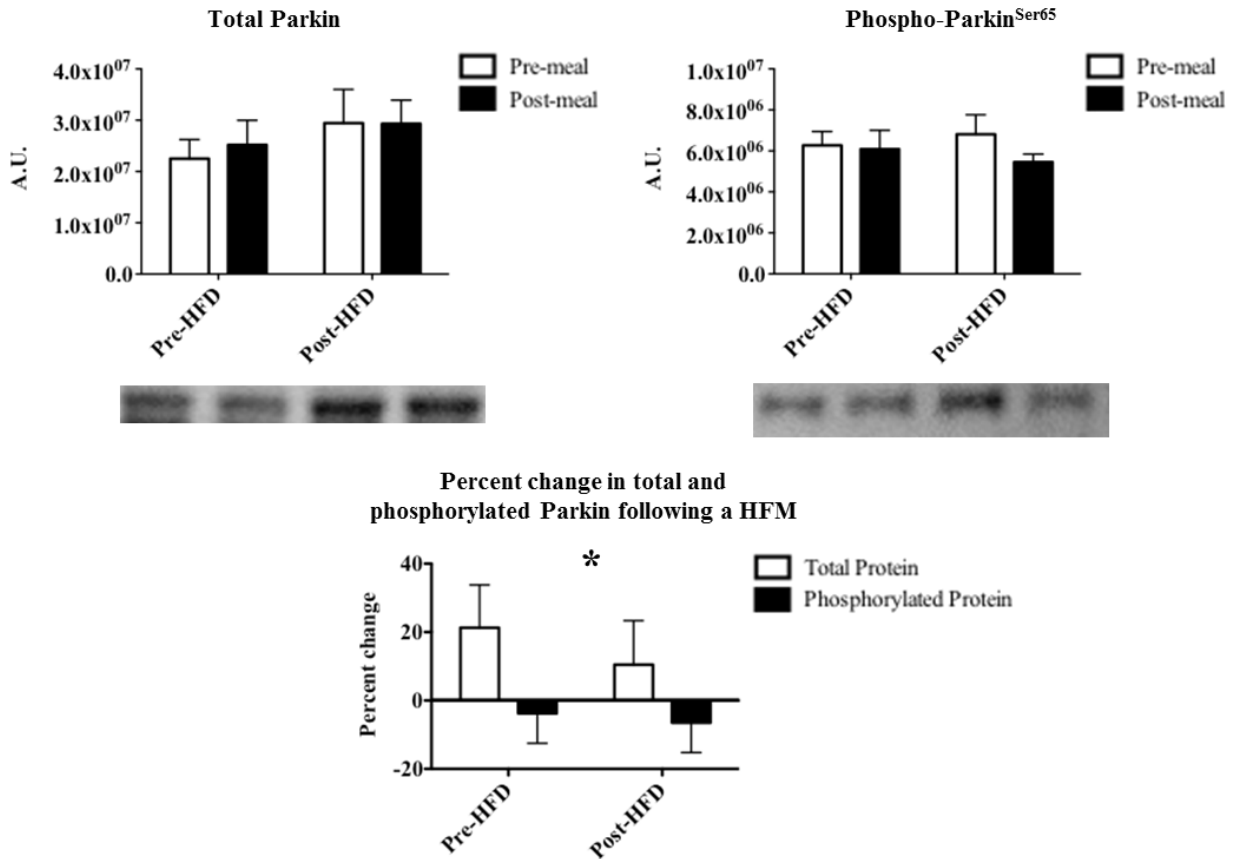


Figure 6. Assessment of changes in skeletal muscle protein content of mitophagy markers. Differences in protein expression of Parkin, a key mitophagy signaling protein. Representative western blots are included below their respective graphs. Data are presented as mean \pm SEM. *, significant HFM effect.

Mitochondrial dynamics and ETC content. Analysis of mitochondrial fusion proteins showed Mfn1 was significantly reduced in response to a HFM. There was no significant response in Mfn2

following a HFM. A 5 day HFD did not significantly affect Mfn1 or Mfn2 in a fasting state or in response to a HFM (figure 7). Mitochondrial fission was assessed via total Drp1 and phospho-Drp1^{Ser616} expression. Total and phosphorylated Drp1 were not significantly altered in response to a HFM. Following a 5 day HFD no change in fasting levels of either total or phosphorylated Drp1 were detected, and the diet did not impact protein content in response to a HFM. Comparison of relative changes in total and phosphorylated Drp1 in response to a HFM before and after a 5 day HFD found no significant differences, suggesting that Drp1, and thus mitochondrial fission was not affected (figure 8).

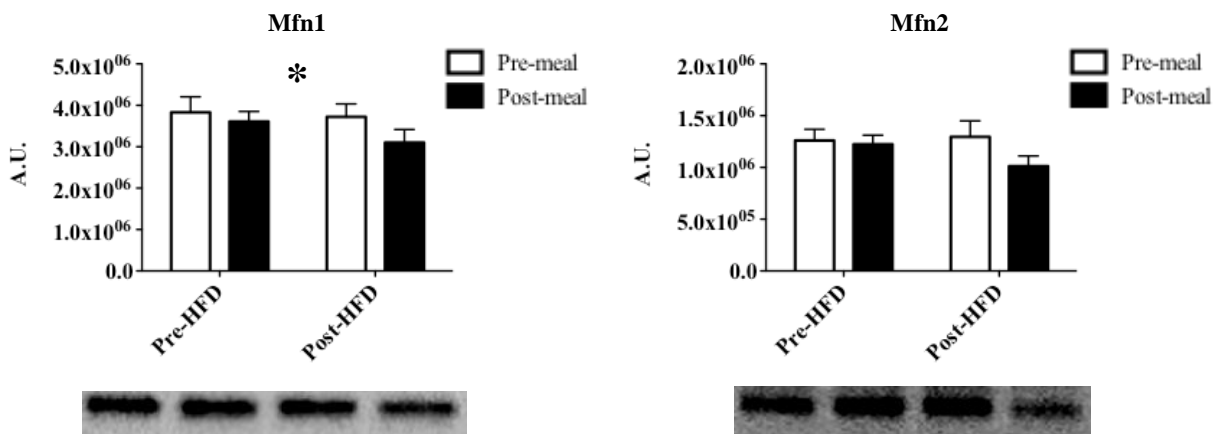


Figure 7. Mitochondrial fusion proteins. Differences in protein expression of mitochondrial fusion proteins in response to a HFM, before and after a HFD. Representative western blots are included below their respective graphs. Data are presented as mean \pm SEM. *, significant meal effect.

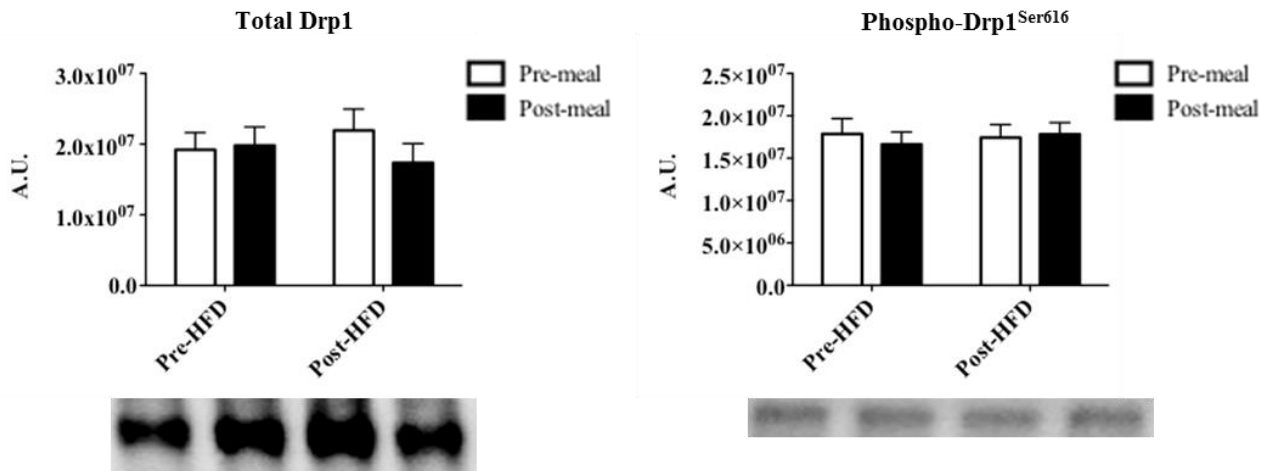


Figure 8. Mitochondrial fission proteins. Differences in protein expression of a key mitochondrial fission protein in response to a HFM, before and after a HFD. Representative western blots are included below their respective graphs. Data are presented as mean \pm SEM.

There were no significant HFD effects on the expression of any ETC complex, or on total ETC content. A significant overall meal effect was found for complex V, which was reduced following a HFM, independent of the HFD. No further meal effects were found, and no significant time point or percent change differences were found for individual complexes or total ETC content (figure 9).

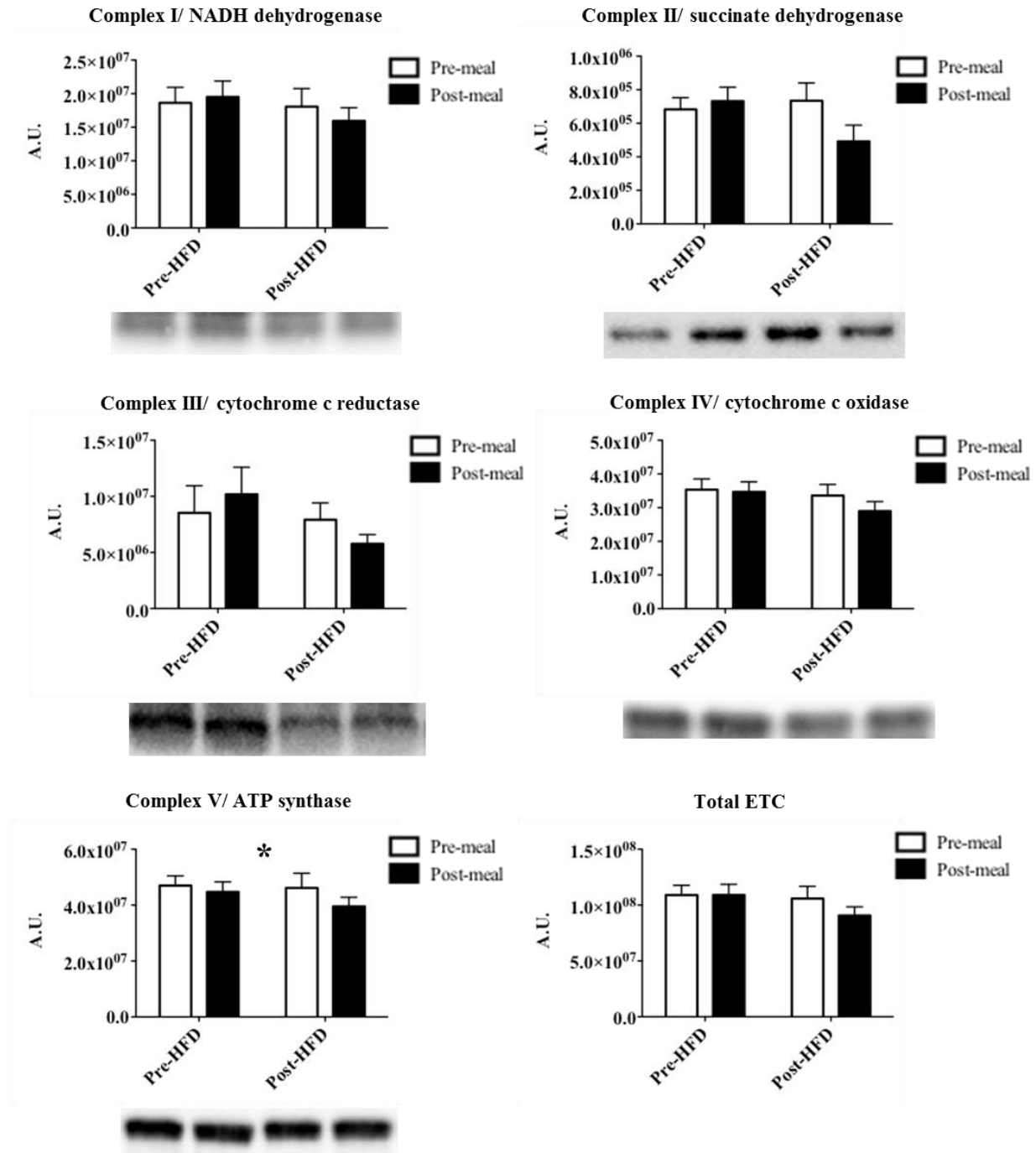


Figure 9. Assessment of ETC protein content. Differences in protein expression of ETC complexes, NADH dehydrogenase, succinate dehydrogenase, cytochrome c reductase, cytochrome c oxidase and ATP synthase in response to a HFM, before and after a HFD.

Representative western blots are included below their respective graphs. Data are presented as mean \pm SEM. *, significant HFM effect.

DISCUSSION

In the present study we investigated how a single HFM and a 5-day HFD affects skeletal muscle autophagy and mitophagy in healthy, sedentary, non-obese males. The autophagy marker LC3 supported a reduction in autophagy, following a HFM. Interestingly, the reduction was exaggerated following a HFM, consumed after the 5-day HFD diet, suggesting an adaptive response. Post-HFD increases in LC3 further support reduction in autophagy because lower autophagic flux reduces the degradation of LC3, and thus an accumulation of LC3 has been suggested to represent diminished autophagic activity (Mizushima, Yoshimori and Levine, 2010). Our data did not unanimously support post-HFD reductions in autophagy, or reductions in response to a HFM following a HFD. The expression of SQSMT1/ p62, which can be inversely correlated to autophagic flux due to lysosomal degradation (Mizushima, Yoshimori and Levine, 2010) did not show a significant accumulation as would be expected.

The current study employed a HFD that supplied participants' with 55% of total calorie intake as fat, and 25% of total calorie intake as saturated fat. Previous studies in the livers of mice (Koga et al., 2010) and immortalized human hepatocytes (Park et al., 2014) have demonstrated that exposure to saturated fatty acids can alter the lipid membrane composition of autophagosomes and lysosomes, inhibiting their fusion and thus blocking autophagy. Whether a 5-day HFD was sufficient to cause similar alterations to the lipid membranes of autophagosomes and lysosomes in

skeletal muscle is not known, but such an alteration would provide a potential mechanism for reduced autophagy following the HFD. Under such conditions we might expect to see an accumulation of LAMP2 and LC3II, but these were not our findings. If data from the present study represents a reduction in autophagy following a HFD, the mechanism may not then be due to inhibited fusion of autophagosomes and lysosomes. Parks et al., (2014) reported finding no accumulation of LC3II in HepG2 cells exposed to palmitate, and that following a failure to fuse with autophagosomes, lysosomes were capable of fusing with endosomes. These data may explain why no differences in LC3II and LAMP2 were reported in the current study, if there was a lipid-induced fault in autophagy. Future studies should therefore aim to isolate and analyze the membrane lipid composition and fusion capabilities of autophagosomes and lysosomes from human skeletal muscle, following a HFD.

We showed no change ULK1 or FoxO3a which would suggest that skeletal muscle autophagy was unresponsive to high-fat treatment. Moller et al., (2015) noted a significant negative correlation between ULK1^{Ser556} and the ratio of LC3II to LC3I in human skeletal muscle of recreationally active males following an acute exercise bout, with the relationship indicative of active autophagy. The current study did not support such a correlation, which may suggest that ULK1 was under the regulatory control of a separate phosphorylation site. Phosphorylation of ULK1 at Ser757, an inhibitory residue, has previously been shown to respond to a prolonged 36h (Moller et al., 2015) and 72h (Vendelbo et al., 2014) fast. ULK1^{Ser757} was also increased during a hyperinsulinemic-euglycemic clamp following a 10h overnight fast, accompanied by a reduced LC3 ratio (Vendelbo et al., 2014). While the clamp is not an accurate simulation of a HFM following an overnight fast, its affect may nevertheless mimic that of a rise in insulin post-HFM. The implied reduction in

autophagy reported by Vendelbo and colleagues (2014) matches the potential reduction in autophagy inferred by LC3 data from the present study. The use of ULK1^{Ser757} either in place of, or in conjunction with ULK^{Ser556} may aid future studies in diagnosing ULK1 activity under fasting and fed conditions.

As discussed, the data could be interpreted as a decrease in autophagy activity. Alternatively, the data may represent an increase in autophagy. Beclin-1 was significantly increased overall following a HFD, which may lend some support to the notion that autophagy was elevated post-HFD. Lira et al., (2013) showed that following 4 weeks of endurance training in mice, basal skeletal muscle autophagy was increased and associated with a small, but significant, increase in beclin-1. Beclin-1 is regulated covalently so it is unclear whether total beclin-1 expression is representative of changes in autophagic flux, particularly as it relates to basal autophagy, which does not depend on Bcl-2/ beclin-1 dissociation (Pattingre et al., 2005). The present study is the first to characterize the skeletal muscle autophagy response to a HFM and HFD in humans. Further studies are required to clarify the findings and resolve the challenges of data interpretation.

The implications of reduced, or increased skeletal muscle autophagy in response to a single HFM for metabolic health are likely minimal and simply representative of a normal metabolic shift between fasting and fed states. A maladaptive response in the autophagy machinery or signaling pathways following a HFD, on the other hand, may have consequences for metabolic wellbeing. He et al., (2012) showed evidence of reduced baseline autophagy using LC3II and p62 degradation following an 8 week HFD. In the same study, He et al., (2012) showed that transgenic mice

incapable of exercise-stimulated autophagy were unable to reverse the negative metabolic impact of a HFD through exercise training. These data raise the question as to whether the consumption of HFDs among a human population have a suppressive effect on autophagy that may impair an individual's ability to restore metabolic function through exercise, thus increasing reliance on pharmacological interventions. Indeed, resistance to exercise has previously been demonstrated in skeletal muscle of patients' with early-onset diabetes (Burns et al., 2007; Hernandez-Alvarez et al., 2010). Transgenic mice with skeletal muscle-specific ablation of autophagy exhibit morphological and functional impairments in mitochondria (Wu et al., 2009; Moresi et al., 2012; Kim et al., 2013), presumably due to reduced mitophagy and a resulting loss of mitochondrial quality control. This remains speculative though due to a dearth of skeletal muscle mitophagy research. Thus, the current study, in addition to autophagy, aimed to examine markers of skeletal muscle mitophagy in response to high-fat feeding.

The process of mitophagy requires the identification and isolation of damaged or dysfunctional organelles' from the mitochondrial reticulum. The depolarization of compromised mitochondria prompts the autophosphorylation of PINK1 at Thr²⁵⁷, stimulating its kinase activity (Kondapalli et al., 2012). A primary target of phospho-PINK1^{Thr257} is Ser⁶⁵ of cytosolic Parkin, which upon phosphorylation, triggers Parkin's E3 ligase activity and mobilization to the outer mitochondrial membrane (Kondapalli et al., 2012; Kane et al., 2014), aided by the receptor action of Mfn2, a mitochondrial fusion protein (Chen and Dorn, 2013). Further interaction with mitochondrial dynamics occurs through the mitochondrial fission protein, Drp1, which is required for mitophagy, seemingly to separate depolarized mitochondria from the wider network (Twig et al., 2008). PINK1 data in the present study indicated a reduction in mitophagy in response to a HFM, after

the 5-day HFD. Although Parkin data supported a reduction in mitophagy post-HFM, the reduction was independent of the HFD, producing conflicting results to those of PINK1 expression.

We are the first group to analyze the protein expression of total PINK1 and Parkin in combination with phosphorylated PINK1 and Parkin in human skeletal muscle. As such, comparative evidence within the literature is unavailable. We have previously studied these proteins in the skeletal muscle of sedentary and endurance-trained males in response to a single HFM (unpublished). Mitophagy markers were significantly elevated in endurance-trained skeletal muscle, but independently of the HFM. However, participants' in the aforementioned study were not subject to the 2-week controlled feeding period implemented in the present study, which may have confounded the outcome. A reduction in mitophagy, particularly in response to a HFM following the 5-day HFD, might be expected if autophagy were similarly reduced, which our data may suggest. Whether these changes are part of a normal adaptive response with protective consequences or a functional impairment is unclear. A consideration as to whether reduced mitophagy is metabolically significant, and if so, what that significance is, depends on the underlying mechanism. Reduced expression of phospho-PINK1^{Thr257} could result from upstream adaptations that alter mitochondrial membrane potential. Alternatively, maladaptations or interference to PINK1-Parkin signaling and/or function following high-fat feeding might be responsible. The HFD employed in the current study is high in saturated fats, which have been shown to increase ROS production in mice (Yuzefovych et al., 2013) and human skeletal muscle (Samocho-Bonet et al., 2012) following a HFD rich in saturated fats. ROS stimulates the transcription of PINK1 (Mei et al., 2009; Priyadarshini, Orosco and Panula, 2013) and Parkin (Zhang et al., 2011), and augments the translocation of Parkin to mitochondria (Joselin et al.,

2012). Increased total PINK1, which was noted following the HFD, may be an indicator of increased ROS-induced transcription. In addition to stimulating transcription and Parkin translocation, ROS can be debilitating if they cause oxidative stress. Parkin for example, through its cysteine side chains, is susceptible to s-nitrosylation and oxidation from ROS, leading to a loss of function (Winklhofer et al., 2003; Chung et al., 2004; Yao et al., 2004, Meng et al., 2011). This may provide a mechanism for mitophagy disruption post-HFD. It is not clear what impact ROS-induced s-nitrosylation and oxidation would have on the expression of mitophagy markers.

Regulation of mitochondrial dynamics is also capable of disrupting mitophagy, indirectly of the PINK1-Parkin axis, via Mfn2 (Chen and Dorn, 2013) and Drp1 (Parone et al., 2008; Twig et al., 2008). The current study showed a HFD-independent reduction in Mfn1 following a HFM, but no change in Mfn2. Both Mfn1 and Mfn2 have previously been shown to remain stable in skeletal muscle of HFD-fed mice, which also displayed increased Drp1 (Jheng et al., 2012). It might be expected that signs of lower mitophagy activity in the current study would be accompanied by a similar shift in Drp1 activity, but this was not the case. Whether the downregulation in Mfn1 is sufficient to shift the dynamic balance toward a state of fission is not clear. The downregulation of ATP synthase in response to a HFM, independent of the HFD, contradicts previous data showing increases in NADH dehydrogenase, succinate dehydrogenase, and ATP synthase following 3 days of high-fat and excess calorie consumption, which returned to baseline upon completion of the full 28-day diet (Samocha-Bonet et al., 2012). Our own unpublished results in sedentary and endurance-trained males showed an overall down-regulation of ETC protein regardless of training status in response to a single HFM, and a significant reduction in cytochrome c oxidase, which was confined to sedentary individuals'. These data maybe significant in the etiology of metabolic

disease, with several studies reporting reduced oxidative phosphorylation proteins in skeletal muscle of obese (Ritov et al., 2010; Wijngaarden et al., 2013), obese early onset diabetics (Hernandez-Alvarez et al., 2010) and obese diabetic patients (Ritov et al., 2010)

The major limitation of the current study was our ability to clearly interpret the autophagy data. There are a number of caveats to the reliability of LC3 measurements due to lysosomal degradation of LC3II, and the cell and stress-specific nature of LC3 production (Klionsky et al., 2012). A reduction in the LC3 ratio could therefore be the result of a rapid turnover in LC3II due to heightened, not reduced, autophagic flux. Similarly, increased total LC3 may represent an upregulation in the cell's autophagic capacity. The use of skeletal muscle biopsy samples, which are static in nature, present an additional challenge when measuring a transient variable such as LC3. While the concurrent analysis of p62 is recommended when interpreting LC3 data (Klionsky et al., 2012), the inverse relationship between p62 and autophagic flux has recently been questioned (Sahani, Itakua and Mizushima, 2014). Furthermore, a lack of change in these autophagy markers may also represent a highly active state of autophagy, but one in equilibrium between protein degradation and production. Accordingly, the analysis of RNA in future studies might benefit the interpretation of protein expression data.

The current study was the first to investigate the effects of a HFM and acute HFD on markers of skeletal muscle autophagy and mitophagy in sedentary humans. We showed evidence of a HFD-dependent reduction in skeletal muscle autophagy and mitophagy following a HFM in sedentary college age males. Nonetheless, our data was insufficient to provide conclusive interpretations of

activity, particularly with regard to the direction of change. Metabolic outcome measures were not analyzed for the present study, although previous investigations into human skeletal muscle and high-fat feeding have noted reduced insulin sensitivity in response to acute lipid infusion (Chavez et al., 2010; Brands et al., 2011) and reduced insulin sensitivity following a 28-day high-fat, high-calorie diet (Samocha-Bonet et al., 2012). Future studies need to combine a greater range of autophagy markers with a wider variety of methods that also examine the relationship to changes in indices of metabolic health. Due to current methodological limitations, it may be necessary to combine protein expression with autophagic assay protocols in primary cell culture and muscle fiber models to produce definitive conclusions about autophagy and mitophagy activity.

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CHAPTER V

Skeletal muscle autophagy and mitophagy in endurance-trained runners, and in response to a high-fat meal

ABSTRACT

We tested the hypothesis that skeletal muscle adaptations to endurance exercise training would enhance autophagy and mitophagy activity leading to greater metabolic flexibility compared to healthy, non-obese sedentary controls. In addition, we aimed investigate whether a high-fat meal elicited different reactions between the two populations', and whether the results were related to enhanced skeletal muscle autophagy and mitophagy. Ten endurance-trained male runners' and nine healthy, sedentary, non-obese males were recruited. Following satisfactory completion of the screening, participants' underwent a maximal oxygen consumption treadmill test to assess VO_{2max} . Participants' later completed a high-fat meal challenge, which consisted of a 12h overnight fast followed by a pre-meal biopsy. Following the biopsy, participant's consumed a high-fat meal and then underwent a second biopsy 4h later. Biopsies were taken from the vastus lateralis and analyzed for protein expression of autophagy and mitophagy markers, and metabolic flexibility. We found no clear evidence of increased autophagic activity between groups' when fasted or following a high-fat meal. Markers of mitophagy were significantly greater in skeletal muscle of endurance-trained individuals' during fasting and following a high-fat meal. Mitophagy markers were not affected by the high-fat meal. Mitophagy markers were positively correlated with VO_{2max} and electron transport chain proteins. No significant differences were found in skeletal muscle metabolic flexibility between groups'. In conclusion, endurance-trained male runners' exhibit elevated mitophagy compared to healthy, sedentary controls independent of a fasting or fed state.

Increases in mitophagy may be the result of greater oxidative capacity and increased oxidative phosphorylation proteins. Elevated mitophagy markers were not associated with notable improvements in metabolic flexibility.

INTRODUCTION

The dramatic increase in the numbers of overweight and obese individuals' over the past few decades is, if uninterrupted, projected to rise to over three billion adults globally by 2030 (Kelly et al., 2008; Ng et al., 2014). Excess body weight is a contributing factor in 90% of type II diabetic patients' (Guh et al., 2009), who are also at increased risk of developing hypertension and cardiovascular disease (Zimmet, Alberti and Shaw, 2001; Sullivan et al., 2005). The onset of T2D is preceded by a marked reduction in skeletal muscle sensitivity a decade or two early, thus representing a major predictor of disease (Lillioja et al., 1988; Warram et al., 1990) and potential pathway for treatment. The precise mechanisms underpinning skeletal muscle insulin resistance are still debated, but a commonly cited cause is the accumulation of lipid metabolites leading to disruption of the insulin signaling cascade (Goodpaster et al., 2001; Itani et al., 2002; Perseghin et al., 2002). In addition, obese, insulin resistance and T2D patients' exhibit markers of impaired mitochondrial function, including lower oxidative capacity (Kelley et al., 2002; Befroy et al., 2007; van de Weijer et al. 2013; Vijgen et al., 2013; Kristensen et al., 2014), reduced transport chain protein content (Hernandez-Alvarez et al., 2010; Ritov et al., 2010; Wijngaarden et al., 2013), gene expression (Mootha et al., 2003; Patti et al., 2003) and activity (Petersen et al., 2004), as well as fewer (Morino et al., 2005; Boushel et al., 2007; Rabol et al., 2009) and smaller (Kelley et al., 2002) mitochondria. Together, these symptoms of obesity may support a scenario in which impaired mitochondrial function leading to reduced and incomplete fatty acid oxidation contributes to the accretion of lipid bodies in skeletal muscle and the subsequent reduction in

insulin sensitivity. Furthermore, short-term high-fat diets, particularly those rich in saturated-fats have been linked to reduced insulin sensitivity (Anderson et al., 2009; Chavez et al., 2010; Samocha-Bonet et al., 2012) in skeletal muscle of healthy adults, which may suggest etiological consequences of habitual high-fat, high-saturated fat diets.

In addition to diet, a sedentary lifestyle is a prominent factor in the etiology of obesity, insulin resistance and T2D (Swinburn et al., 2004; Kaur, 2014). Conversely, exercise training is associated with reduced body mass index (BMI) (Marandi et al., 2013) and increased insulin sensitivity in healthy adults (Dela et al., 1992; Houmard et al., 1993; Evans et al., 2001), as well as improved insulin sensitivity in obese (Goodpaster, Katsiatas and Kelley, 2003; Shojaae-Moradie et al., 2007) and T2 diabetic patients' (Dela et al., 1995). Exercise is also a modulator of autophagy in skeletal muscle (Jamart et al., 2012a and 2012b; Moller et al., 2015). Autophagy is a process of lysosomal protein degradation, including organelles such as mitochondria (Rikka et al., 2011). The term mitophagy was coined by Lemasters (2005) to describe mitochondrial-specific autophagy, which is required for the maintenance of mitochondrial quality control (Gottlieb and Carreira, 2010; Patel, Shirihai and Huang, 2013). Chronic exercise training leads to increased markers of basal autophagy and mitophagy in mice (Lira et al., 2013), conversely, a high-fat diet has a suppressive effect on autophagy in murine skeletal muscle (He et al., 2012). It is unknown if human skeletal muscle responds similarly. Endurance training has been shown to enhance mitochondrial performance with associated improvements in metabolic flexibility and insulin sensitivity in response to lipid overload (Dube et al., 2015), an antagonist of skeletal muscle insulin sensitivity. The significance of basal autophagy and mitophagy activity is unclear, but if greater in endurance-trained skeletal muscle, it may support a healthier mitochondrial population that ultimately

increases skeletal muscle capacity to withstand high-fat metabolic insults. The current study will therefore test the hypothesis that endurance-trained individuals' exhibit greater autophagy and mitophagy activity markers compared to sedentary individuals'. We will also investigate whether training status influences the autophagy and mitophagy response to a high-fat meal, and if differences are associated with improved skeletal muscle metabolic flexibility.

MATERIALS AND METHODS.

Participants'. Nine healthy, non-obese, sedentary (< 2 days, 20 min/day of low-intensity physical activity) males and 10 endurance-trained (≥ 5 -h running per week, and 2 competitions in the past 12 months) male runners aged 18-45 years completed the study. Participants' were weight stable (± 2.5 kg) for the past 6 months with a BMI > 18 or < 30 kg/m², and were not under the influence of any medications or supplements known to affect study measures. All participants' had blood pressure < 140/90 mmHg, fasting glucose < 126 mg/dL, total cholesterol < 240 mg/dL or triglycerides < 300 mg/dL, and percentage of habitual calorie intake composed of < 40% fat and < 15% saturated fat. Participants' were non-smokers with no family history of disease. All study procedures were approved by the Virginia Polytechnic Institute and State University Institutional Review Board. Prior to participation all procedures, benefits and any potential risks associated with the study were explained to participants' before written consent was provided.

Experimental design. Following successful completion of screening procedures, all participants' underwent a maximal treadmill test to volitional exhaustion to determine maximal oxygen consumption (VO_{2max}). No less than 36-h following the treadmill test, participants' completed a high-fat meal (HFM) challenge. Muscle biopsies were taken immediately prior to, and 4-h after an HFM for assessment of skeletal muscle autophagy, mitophagy, and metabolic flexibility. A schematic of the design is presented in figure 1.

HFM challenge. Participants' arrived at the laboratory following a 12-h overnight fast. Biopsies of the vastus lateralis muscle were taken before and 4 hours after a high-fat meal. A HFM consisted of two Jimmy Dean sausage, egg and cheese biscuits containing 768 kcal, composed of 53.9 g fat (25.5 g saturated fat), 44.2 g carbohydrate and 25.7 g protein. Participants' were required to consume the HFM within 10 minutes and remain seated and awake for the duration of the meal challenge. Following the initial biopsy and prior to the HFM, participants' were fitted with an intravenous catheter in the antecubital vein for baseline and hourly blood sampling. Pre and post biopsies were taken from separate legs.

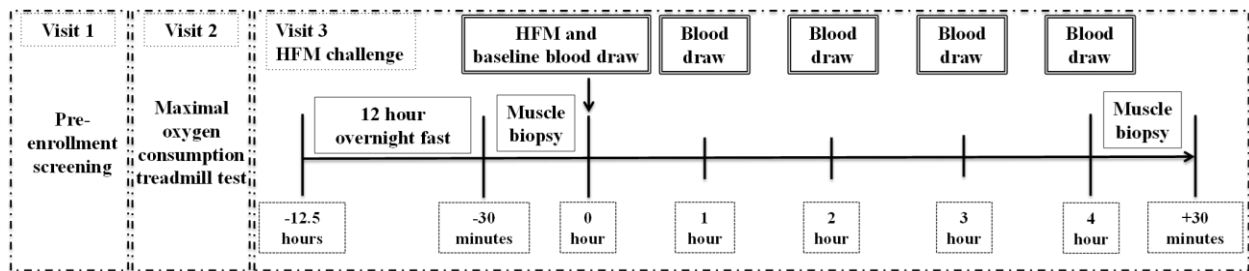


Figure 1. Schematic of study design. Participants' completed a pre-enrollment screening prior to completing a maximal oxygen consumption test. Participants' fasted for 12-h overnight prior to a baseline skeletal muscle biopsy taken at least 36-h after last exercise bout. Participants' consumed a HFM and rested for 4-h before completing a second follow-up skeletal muscle biopsy. Blood was drawn every hour. HFM, high-fat meal.

Measurements.

Body mass and composition. Body weight was measured to the nearest ± 0.1 kg on a digital scale (Model 5002, Scale-Tronix, White Plains, NY). Height was measured to the nearest ± 0.1 cm using

a stadiometer. Body composition (total fat and fat-free mass) was analyzed by dual-energy x-ray absorptiometry (General Electric, Lunar Digital Prodigy Advance, software version 8.10e Madison, WI).

Maximal Oxygen Consumption. A continuous incremental treadmill exercise test to volitional exhaustion was used to assess maximal oxygen consumption ($\text{VO}_{2\text{max}}$). Tests were performed on a motorized treadmill (Q-Stress TM65, Quinton, Bothell, WA) operated with Q-stress Exercise Test Monitor software (v3.5, Quinton). Participants' ran at a pace equivalent to 70% of predicated maximum heart rate (220-age) at 0% grade (Fox, Naughton and Haskell, 1971). The treadmill grade was increased 2.5% every 2 min until volitional exhaustion. Respiratory data was recorded throughout the exercise test using a ParvoMedics gas analyzer (model TrueOne 2400, ParvoMedics, Sandy, UT) and TrueOne OUSW 4.3.4 data acquisition and analysis software (v20111228, ParvoMedics). Achievement of maximal oxygen consumption was confirmed through meeting three of the following four criteria: 1) a plateau in VO_2 ($< 0.15 \text{ l}\cdot\text{min}^{-1}$ over final minute); 2) a respiratory exchange ratio of > 1.05 ; 3) a maximal test heart rate ± 10 bpm of age-predicated maximum heart rate; 4) a rating of perceived exertion of 19 or 20 on the Borg scale (Borg, 1982).

Muscle biopsies. Biopsies were taken from the vastus lateralis muscle using a suction-modified Bergstrom needle technique (Bergstrom, 1962). An area of skin in the region of the vastus lateralis was shaven and cleansed with a povidine-iodine solution. The skin, adipose tissue and skeletal muscle fascia was anesthetized using 10mL lidocaine (1%). The skin was incised (0.75 cm) with a #10 scalpel, and the fascia fibers were separated with the blunt edge of the scalpel. The Bergstrom needle (5 mm) was inserted into the vastus lateralis and suction applied. Muscle used to assess pyruvate dehydrogenase (PDH) activity and metabolic flexibility was immediately placed in SET

buffer (0.25 M Sucrose, 1 mM EDTA, 0.01 M Tris-HCl and 2 mM ATP) and stored on ice until homogenization (~25 min). Muscle tissue used for western blotting was placed in ice-cold cell lysis buffer (50 mM Tris-HCl, EDTA 1 mM, NaCl 150 mM, SDS 0.1%, sodium deoxycholate 0.5%, igepel Ca 630 1%, pH 7.5) with halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Pittsburg, PA), and 0.1 mM bafilomycin A (Invivogen, San Diego, CA), then snap-frozen in liquid nitrogen. Protein samples were stored at -80°C for later analysis.

Muscle homogenization and western blot analysis. Frozen muscle tissue samples were homogenized in ice-cold lysis buffer containing 0.1 mM bafilomycin A1 in a Bullet Blender Homogenizer (Next Advance, NY) using 1.0mm Zirconium Oxide beads (Next Advance). Samples were further homogenized with a 23-gauge needle, and centrifuged at 14,000 g for 15 min at 4°C to remove insoluble components. Supernatant protein concentrations were determined spectrophotometrically using the bicinchoninic acid assay (BCA) (Thermo Scientific). Lysis buffer was added to samples for adjustment to equal concentrations and combined with equal volumes 2 x Laemelli buffer and heated for 5 min at 95°C. Equal amounts of protein were separated on SDS-PAGE gels (Criterion TGX Stain-Free Gels, Bio-Rad, Hercules, CA), which were activated via ultra violet light exposure (ChemiDoc Touch Imaging System, Bio-Rad) prior to transfer. Proteins were transferred to PVDF membranes using a Trans-Blot Turbo Transfer System (Bio-Rad), which were then imaged (Bio-Rad) for quantification of total lane protein. PVDF membranes were blocked for 1-h at room temperature in 5% non-fat dry milk or 5% bovine serum albumin prior to overnight incubation at 4°C with primary antibodies. Membranes were probed with primary antibodies against FoxO3a (cat# 17026), LAMP2 (cat# 25632), MFN1 (cat# 57602), MFN2 (cat# 56889), Parkin (cat# 15954), PINK1 (cat# 23707) (Abcam, Cambridge, MA), Beclin-1 (cat# 3738), Bcl-2 (cat# 2870s), LC3B (cat# 2775), ULK1 (cat# 4773) (Cell Signaling, Danvers,

MA), total OXPHOS cocktail (cat# MS601) (MitoSciences, Eugene, OR), DRP1 (cat# 110-55288) (Novus Biologicals, Littleton, CO), SQSMT1/ p62 (cat# 28359) (Santa Cruz Biotechnology, Dallas, TX) and phospho-specific primary antibodies against FoxO3a (Thr³², cat# 26649), Parkin (Ser⁶⁵, cat# 154995) (Abcam), DRP1 (Ser⁶¹⁶, cat# 3455), ULK1 (Ser⁵⁵⁵, cat# 5869 – equivalent to human Ser⁵⁵⁶) (Cell Signaling), and PINK1 (Thr²⁵⁷, cat# 68-0057-100) (Ubiquigent, Dundee, Scotland, UK). Following primary antibody incubation, membranes were incubated for 1-h at room temperature with HRP-conjugated anti-mouse, anti-rabbit, anti-sheep (Jackson Immuno Research Laboratories, West Grove, PA) and anti-goat (Santa Cruz) secondary antibodies. Proteins were visualized via chemiluminescence (Clarity Western ECL Substrate, Bio-Rad, or SuperSignal West Femto, Thermo Scientific) and quantified using Image Lab Software (v5.2.1, BioRad) and normalized to total lane protein content. Molecular weight was determined by Precision Plus Protein Unstained Standards (Bio-Rad).

Metabolic flexibility. Metabolic flexibility was assessed by measuring [1-14C] pyruvate oxidation \pm non-labeled BSA (0.5%) bound-palmitate. Flexibility is denoted by the percentage decrease in pyruvate oxidation in the presence of free fatty acid (e.g. a higher percentage is indicative of greater metabolic flexibility). It is expressed as the ratio of CO₂ production with labeled pyruvate over CO₂ production with labeled pyruvate in the presence of palmitate. Each skeletal muscle sample was minced ~200 times with scissors, transferred to a glass homogenization tube and homogenized on ice using a Teflon pestle (12 passes at 150 RPM). The sample rested on ice for ~30 sec and the homogenization steps were repeated. The homogenate was transferred to an Eppendorf tube and fresh sample was used to measure pyruvate oxidation. Briefly, 80 μ L of a 20-fold (wt:vol) diluted muscle homogenate was incubated with 320 μ L of reaction media (pH 7.4). Final concentrations of the reaction media were in mmol per liter: sucrose, 100; Tris-HCl,

10; potassium phosphate, 5; potassium chloride, 80; magnesium chloride, 1; L-carnitine, 2; malate, 0.1; ATP, 2; coenzyme A, 0.05; dithiothreitol, 1; EDTA, 0.2; and bovine serum albumin, 0.3%. After 1hr of incubation at 37°C, 200 µl of 45% perchloric acid was injected to stop the reaction and evolve $^{14}\text{CO}_2$ from the reaction media. $^{14}\text{CO}_2$ produced during the incubation was trapped in 400 uL of 1 M sodium hydroxide. Trapped $^{14}\text{CO}_2$ was determined by liquid scintillation counting by use of 5 ml EcoLite liquid scintillation cocktail (MP Biomedicals, Santa Ana, CA) on the LS 6500 scintillation counter (Beckman Coulter, Pasadena, CA). Homogenate protein concentrations were determined spectrophotometrically using a BCA assay (Thermo Scientific).

Statistical analysis. Two-way repeated measures analysis of variance was used to determine differences between and within groups at pre and post-HFM time points for protein and metabolic outcome measures. Multiple comparisons were performed using a Tukey post-hoc analysis. Independent t-tests were used to compare group characteristics and percent change in protein levels from pre and post-meal time points between groups. Correlations were examined via multivariate analysis. Data that did not follow a normal distribution were log base 10, or square root transformed. All protein expression data were analyzed with $n = 9$ endurance-trained participants' and $n = 9$ sedentary participants'. Participant characteristics, PDH activity and metabolic flexibility represent data for $n = 10$ endurance-trained participants' and $n = 9$ sedentary participants'. All data are expressed as means \pm standard error of the mean (SEM). The significance level was set *a priori* at $\alpha = .05$.

RESULTS

Participant characteristics. Participant characteristics can be found in table 1. No significant differences in age, body mass, BMI or fasting glucose between sedentary or endurance-trained groups'. Endurance-trained participants' demonstrated a significantly lower body fat percentage

and fasting blood triglycerides compared to sedentary participants'. In addition, endurance-trained participants' showed a significantly greater VO_{2max} compared to sedentary participants'.

Table 1. Participant characteristics.

Characteristics	Sedentary	Endurance
Age (yrs)	23.2 ± 1.3	26.5 ± 2.5
Body mass (kg)	76.4 ± 4.7	71.0 ± 1.7
BMI	24.6 ± 1.0	22.5 ± 0.8
Body fat percentage	26.3 ± 3.0	14.4 ± 1.3*
VO_{2max} (ml·kg·min ⁻¹)	43.1 ± 3.4	65.8 ± 2.3*
Fasting glucose (mg·dL ⁻¹)	86.9 ± 2.2	79.1 ± 4.8
Fasting triglycerides (mg·dL ⁻¹)	103.1 ± 13.7	68.3 ± 7.4*

Values are mean ± SEM. *, $p < 0.05$.

Autophagy markers. There was no difference in the ratio of LC3II and LC3I between groups before or after the HFM challenge. Similar results were found for the expression of SQSMT1/ p62 (figure 2). Expression of LC3II was not significantly different between groups', or affected by a HFM. There was a significant group effect for greater total LC3 content in endurance-trained participants'. Total LC3 content trended higher pre-meal ($p = 0.081$), and was significantly greater post-meal. Analysis of the percent change in LC3II/ LC3I ratio, SQSMT1/p62, LC3II and total LC3 following a HFM confirmed no difference in response to a HFM between groups'. Expression of the lysosome marker, LAMP2, was also similar between groups' and unresponsive to a HFM. However, analysis of the percent change between groups in response to a HFM revealed a trend ($p = 0.057$) toward greater LAMP2 post-meal in the endurance-trained group ($18.5 \pm 3.3\%$) compared to sedentary group ($-2.6 \pm 1.6\%$). In addition, expression of the anti-autophagy protein,

Bcl-2, and pro-autophagy protein, Beclin-1, were measured, but showed no significant differences between groups' or in response to a HFM (figure 3).

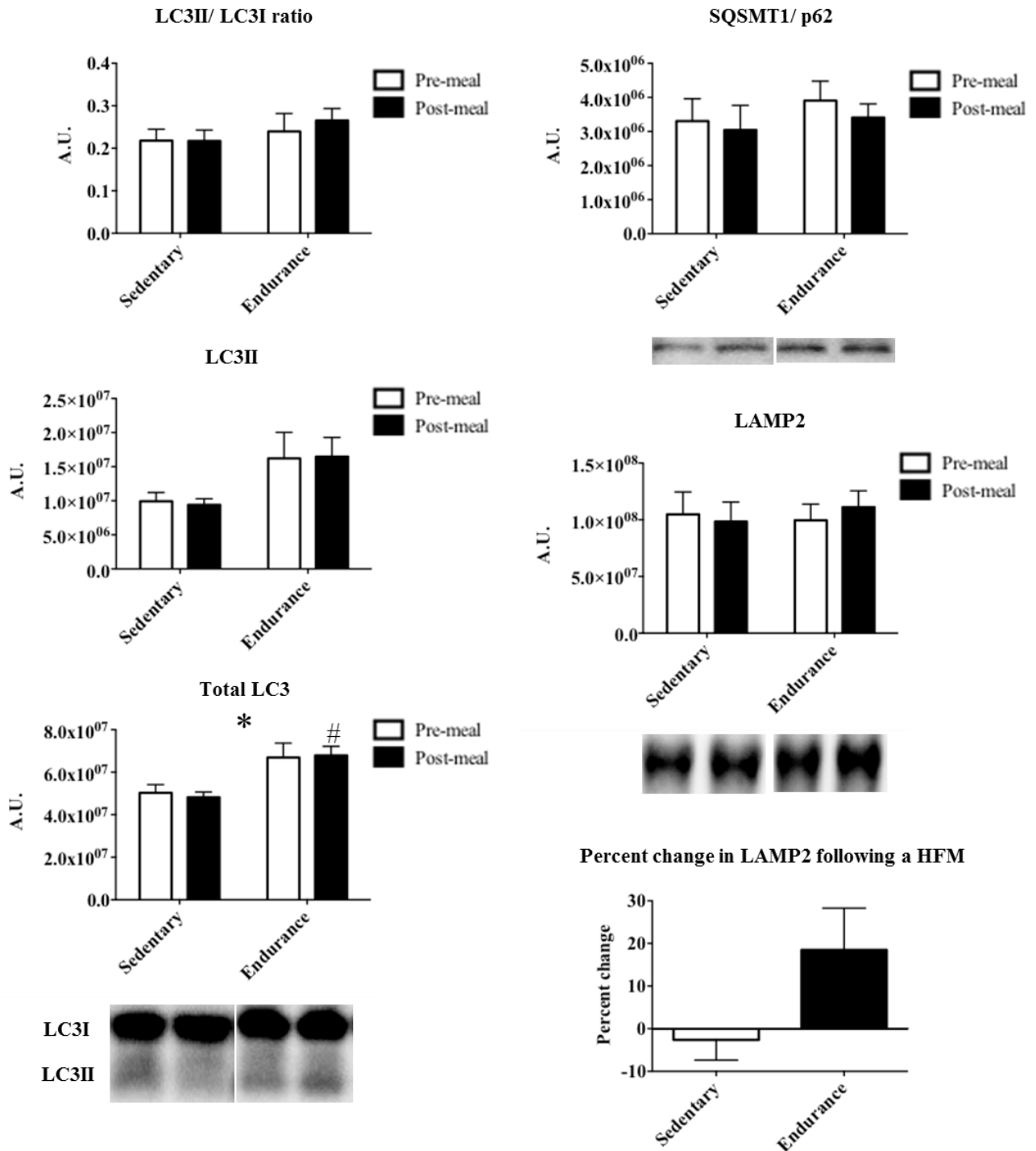


Figure 2. Assessment of markers of skeletal muscle autophagy activity. Differences in markers of autophagy activity, LC3 ratio, SQSMT1/ p62, LC3II, total LC3 and LAMP2 between sedentary and endurance-trained participants' before and after a HFM. Representative western blots are included below their respective graphs. Data are presented as mean \pm SEM. *, significant overall group difference; #, significant time-point difference between groups'.

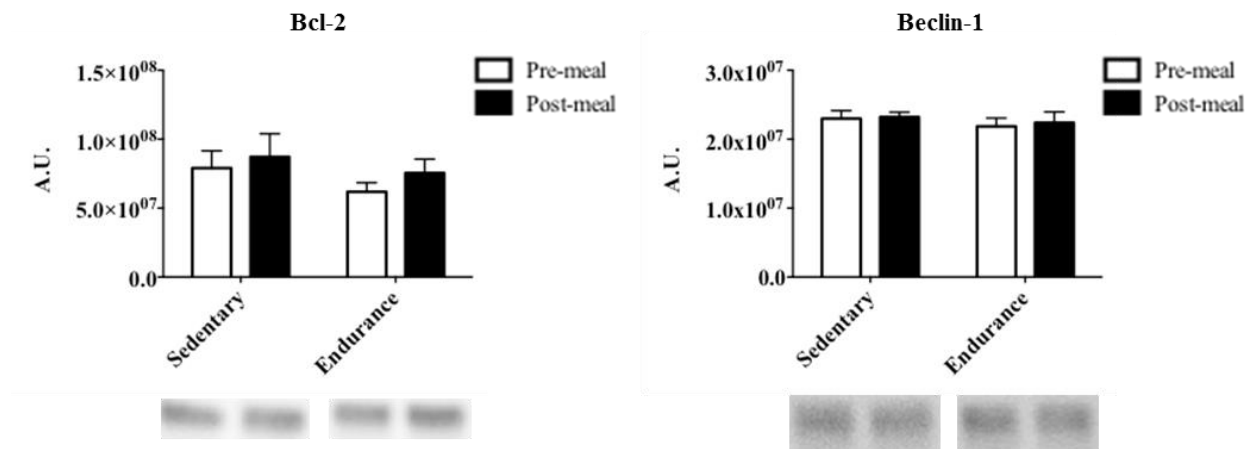


Figure 3. Skeletal muscle protein content of autophagy regulators. Differences in anti-autophagy, Bcl-2, and pro-autophagy, Beclin-1, content before and after a HFM in sedentary and endurance-trained participants'. Representative western blots are included below their respective graphs. Data are presented as mean \pm SEM.

Analysis of skeletal muscle total ULK1 content revealed no significant group or meal effects, although there was a trend for reduced total ULK1 content overall post-meal ($p = 0.089$). Similarly, no significant differences in phospho-ULK1^{Ser556} were observed, but a trend existed for reduced ULK1^{Ser556} overall post-meal ($p = 0.072$). There was also no significant difference in the observed

phosphorylation status of ULK1 (figure 4). Skeletal muscle FoxO3a content was significantly greater in endurance-trained participants' compared to sedentary participants' overall. The group difference was partially the product of a trend toward greater FoxO3a content in endurance-trained skeletal muscle post-meal ($p = 0.073$), although no significant meal effect was found. Phospho-FoxO3a^{Thr32} was not significantly different between groups' or following a HFM (figure 5). These data demonstrate greater expression of total FoxO3a relative to phospho-FoxO3a^{Thr32} in endurance trained participants' compared to sedentary participants', particularly post-meal, implying reduced FoxO3a activity in the endurance-trained group following a HFM compared to the sedentary group. Comparison of the percent difference between the endurance-trained and sedentary groups' in total FoxO3a and phospho-FoxO3a^{Thr32} support this finding (figure 5). The percent difference in FoxO3a and FoxO3a^{Thr32} between endurance-trained and sedentary groups' was significant overall, and following a HFM, suggesting a lower phosphor-FoxO3a^{Thr32} status in the endurance-trained group.

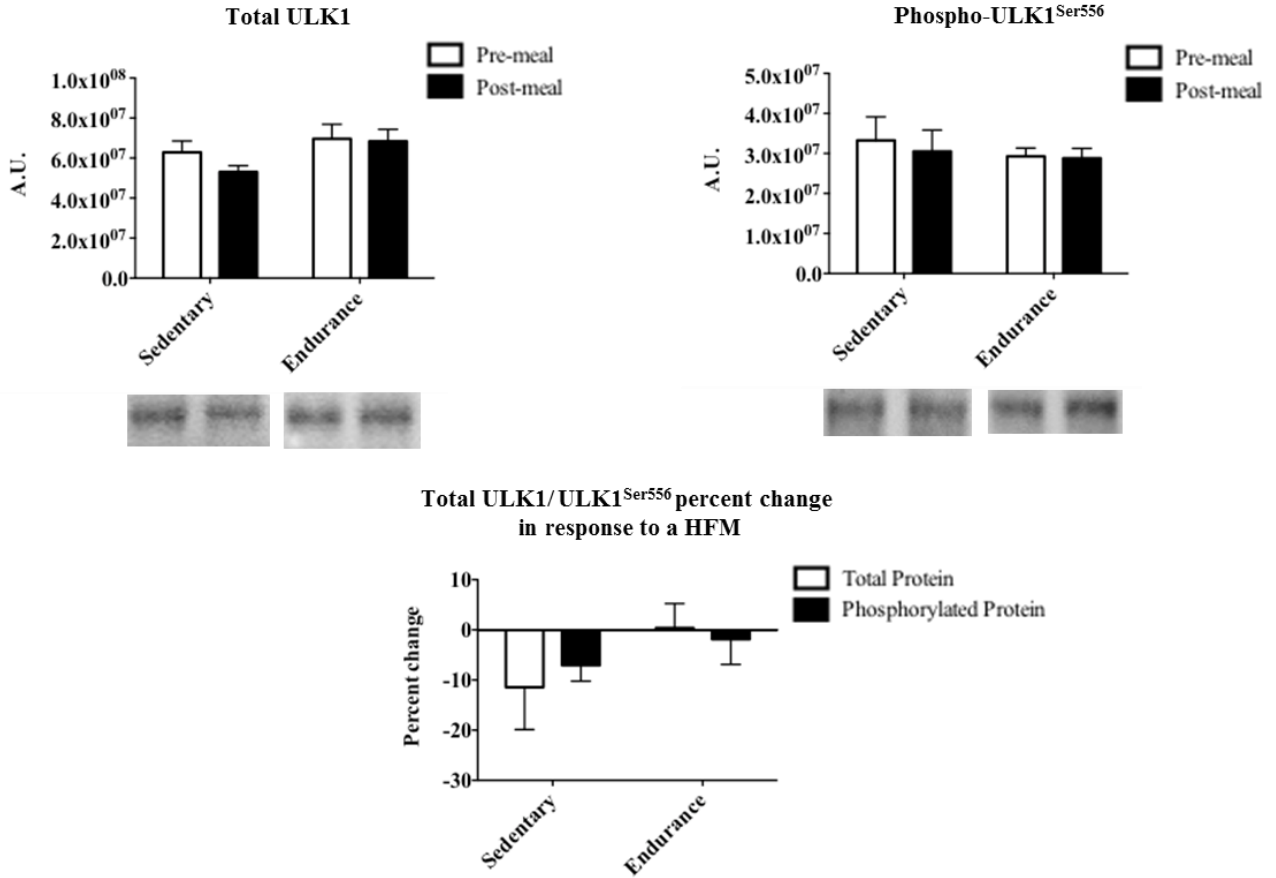
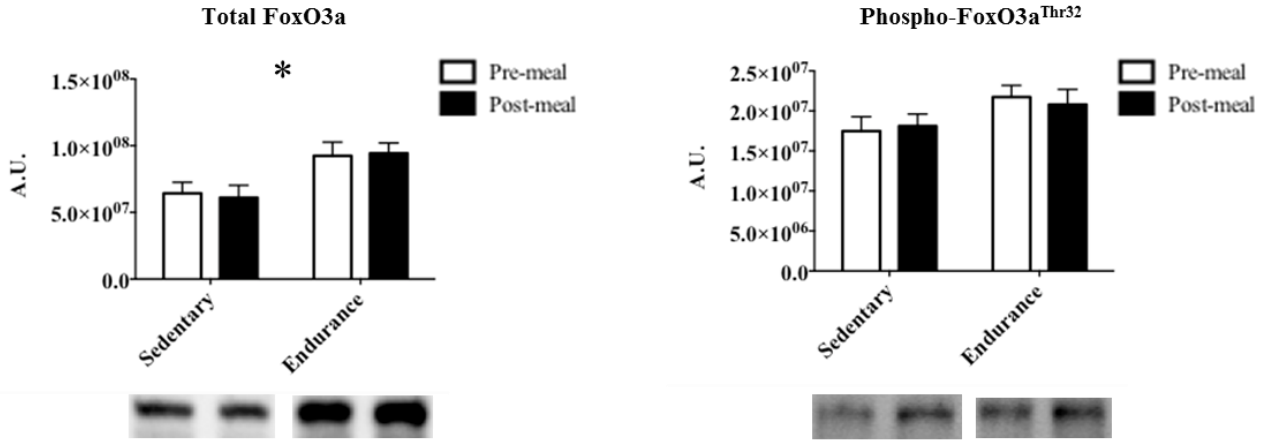


Figure 4. Assessment of changes in skeletal muscle total ULK1 and phospho-ULK^{Ser556}. Differences in total ULK1 and phospho-ULK1^{Ser556} before and after a HFM in endurance-trained and sedentary participants', and percent change in total ULK1 and phospho-ULK1^{Ser556} following a HFM. Representative western blots are included below their respective graphs. Data are presented as mean ± SEM.



Total FoxO3a/FoxO3a^{Thr32} percent difference between endurance-trained and sedentary participants

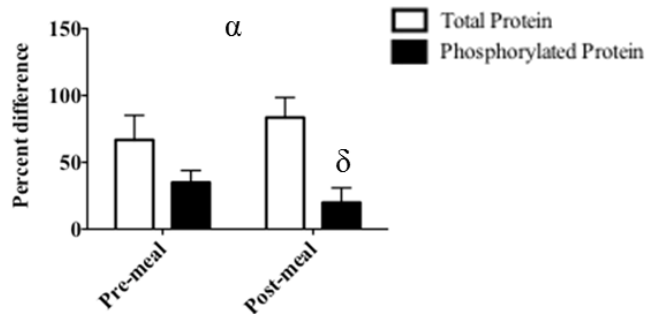


Figure 5. Assessment of changes in skeletal muscle total FoxO3a and phospho-FoxO3a^{Thr32}. Differences in total FoxO3a and phospho-FoxO3a^{Thr32} before and after a HFM in endurance-trained and sedentary skeletal participants', and percent difference in total FoxO3a and phospho-FoxO3a^{Thr32} between endurance-trained and sedentary participants' at pre and post-meal time points. Representative western blots are included below their respective graphs. Data are presented as mean ± SEM. *, significant overall group difference; α, significant overall difference between total and phosphorylated protein; δ, significant time point difference between total and phosphorylated protein.

Mitophagy markers. Expression of total PINK1 was not significantly different between groups', and was unaffected by a HFM. There was however a significant group effect for greater phospho-PINK1^{Thr257} content in endurance-trained participants' compared to sedentary participants'. PINK1^{Thr257} was also significantly greater in skeletal muscle of endurance-trained participants' before and after a HFM. These data suggest a higher rate of phosphorylation and thus PINK1 activity in skeletal muscle of endurance-trained participants' (figure 6). Total Parkin content was significantly greater overall in the skeletal muscle of sedentary participants'. Total Parkin was not significantly different pre-meal, but was significantly greater in sedentary participants' post-meal. Conversely, phospho-Parkin^{Ser65} was significantly greater in skeletal muscle of endurance-trained participants' pre and post-meal. Similarly to PINK1, these results suggest a greater proportion of Parkin is phosphorylated in skeletal muscle of endurance-trained participants', indicating higher protein activity (figure 6).

These data are supported by analysis of percent differences between endurance-trained and sedentary groups', which found relative differences in total PINK1 and Parkin did not compensate for significantly greater relative differences in phospho-PINK1^{Thr257} and phospho-Parkin^{Ser65}, pre and post-meal (figure 7). Combined, these data may imply greater mitophagic activity in skeletal muscle of endurance-trained participants'. Comparison of post-meal percent changes for total PINK1 and phospho-PINK1^{Thr257}, and total Parkin and phospho-Parkin^{Ser65} did not reveal any significant change in the relationship between total and phosphorylated PINK1 and Parkin following a HFM, within or between groups'. These data may suggest that mitophagy activity is not significantly altered by a HFM, independent of training status.

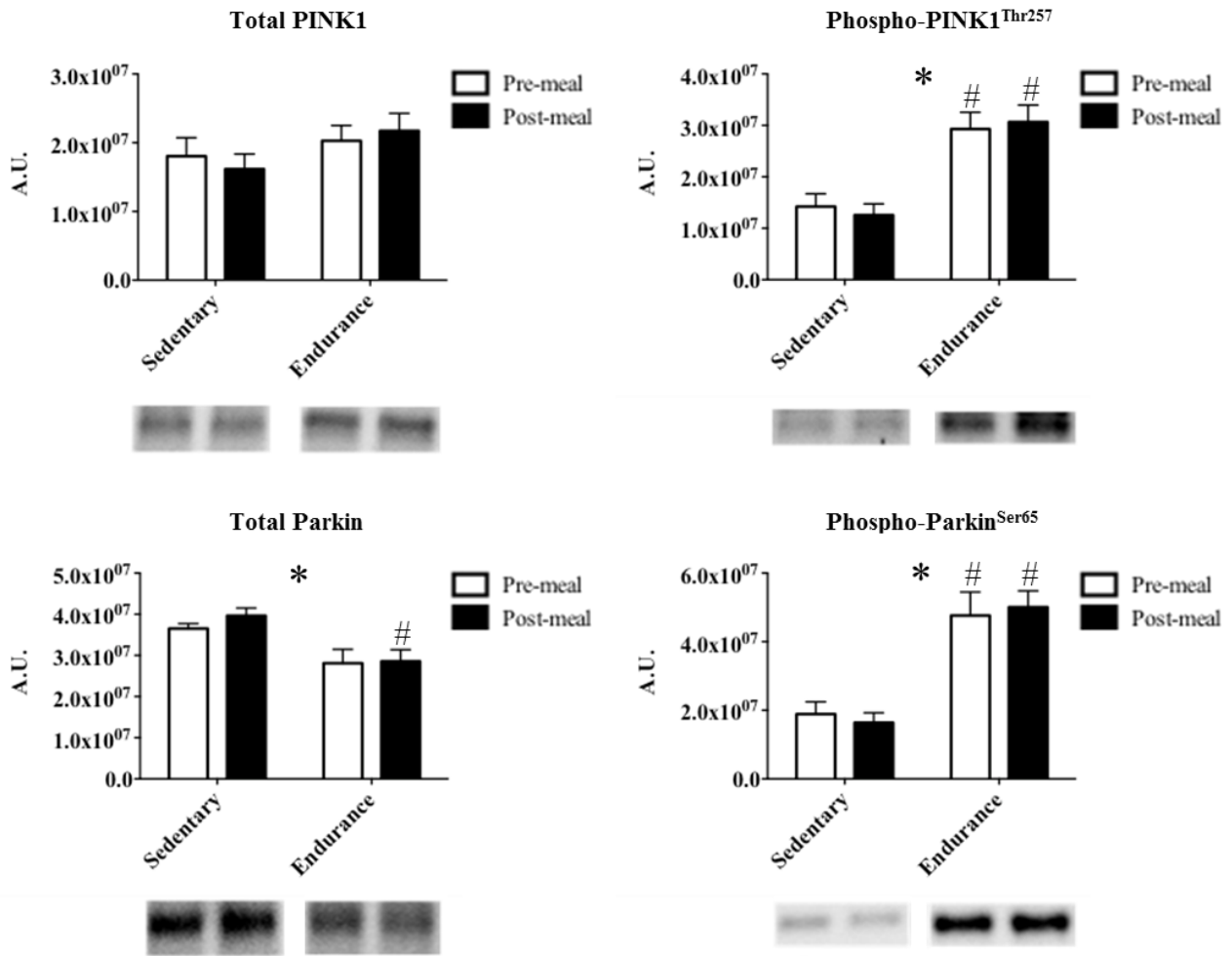
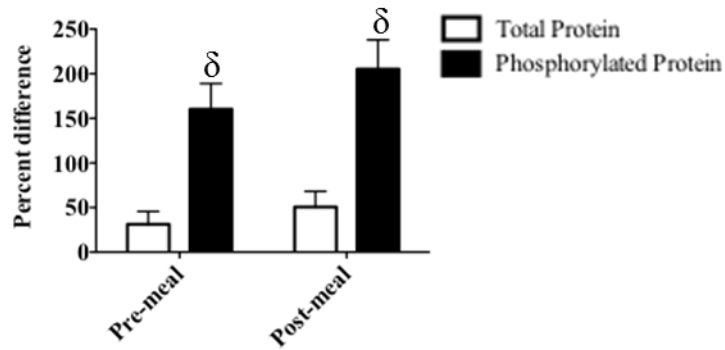


Figure 6. Assessment of changes in skeletal muscle protein content of mitophagy markers. Differences in total PINK1 and phospho-PINK1^{Thr257}, and total Parkin and phospho-Parkin^{Ser65} protein content between endurance-trained and sedentary participants' at pre and post-meal time points. Representative western blots are included below their respective graphs. Data are presented as mean ± SEM. *, significant overall group difference; #, significant time-point difference between groups'.

Total PINK1/ PINK1^{Thr257} percent difference between endurance-trained and sedentary participants



Total Parkin/ Parkin^{Ser65} percent difference between endurance-trained and sedentary participants

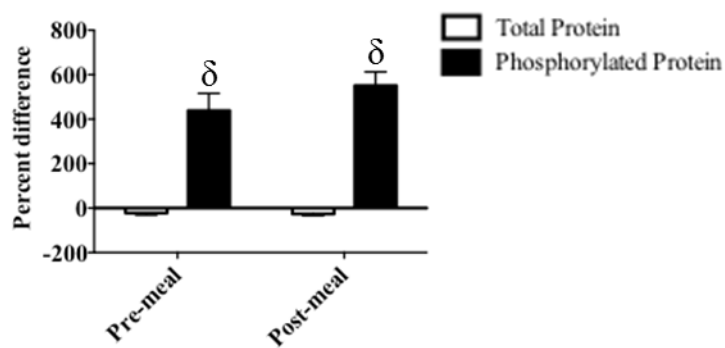


Figure 7. Assessment of relative differences in skeletal muscle mitophagy activity markers. Percent differences between endurance-trained and sedentary participants' for total PINK1 and phospho-PINK1^{Thr257}, and total Parkin and phospho-Parkin^{Ser65} at pre and post-meal time points. Data are presented as mean ± SEM. δ, significant time point difference between total and phosphorylated protein.

Mitochondrial dynamics and ETC content. Analysis of mitochondrial fusion proteins found no significant differences in skeletal muscle Mfn1 content between groups' or in response to a HFM.

Skeletal muscle Mfn2 content revealed a significant group effect for greater protein in endurance-trained participants'. Mfn2 expression trended higher in endurance-trained participants' pre-meal ($p = 0.071$) and was significantly greater post-meal (figure 8). Analysis of mitochondrial fission protein, Drp1, found significantly greater expression of total Drp1 in endurance-trained skeletal muscle overall. Total Drp1 was not significantly different either pre or post-meal between groups', but did trend higher in endurance-trained participants' post-meal ($p = 0.062$). Phospho-Drp1^{Ser616} content was significantly greater overall in skeletal muscle of endurance-trained participants', trending higher pre-meal ($p = 0.061$), and significantly higher post-meal. No within groups' differences were found following a HFM, although a trend for reduced phospho-Drp1^{Ser616} was found in sedentary participants' following a HFM ($p = 0.79$), producing a significantly different percent change in phospho-Drp1^{Ser616} between endurance-trained ($2.8 \pm 2.0\%$) and sedentary participants' ($-14.2 \pm 1.7\%$) following a HFM (figure 8). No such effect was found for total Drp1, indicating potentially higher Drp1 activity post-meal in the endurance-trained group relative to the sedentary group. Analysis of percent differences between endurance-trained and sedentary participants' found a significantly greater phosphorylation status overall in endurance-trained skeletal muscle, indicating higher protein activity and possibly greater mitochondrial fission. Percent differences in total and phosphorylated Drp1 between endurance-trained and sedentary groups' were not significantly different pre-meal, but were significant post-meal, supporting a proportionally higher phosphorylation status and protein activity following a HFM in endurance-trained participants' (figure 9).

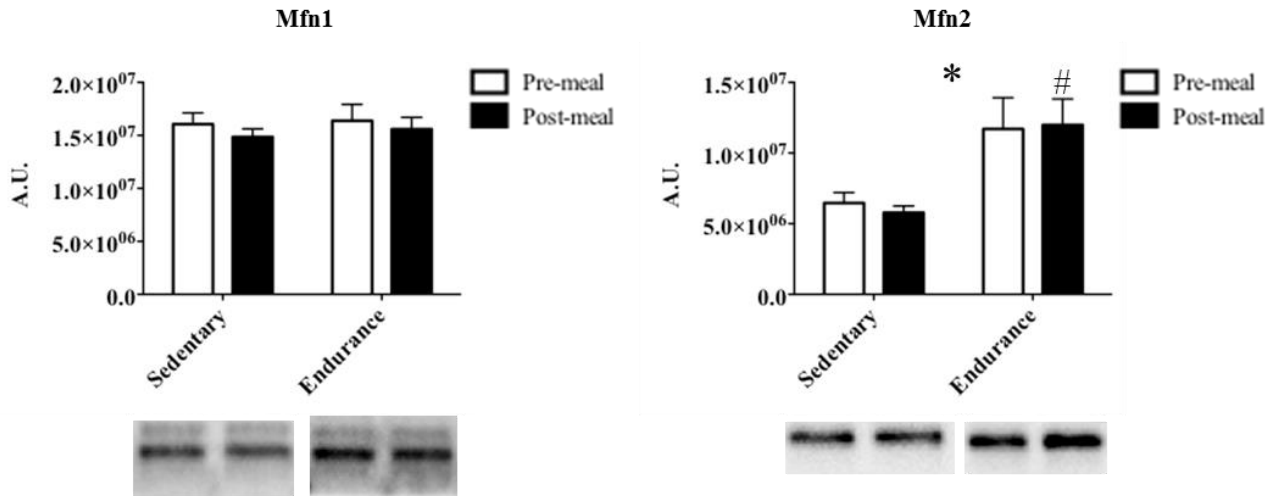


Figure 8. Assessment of skeletal muscle mitochondrial fusion markers. Differences in Mfn1 and Mfn2 between endurance-trained and sedentary participants' at pre and post-meal time points. Representative western blots are included below their respective graphs. Data are presented as mean \pm SEM. *, significant overall group difference; #, significant time-point difference between groups'.

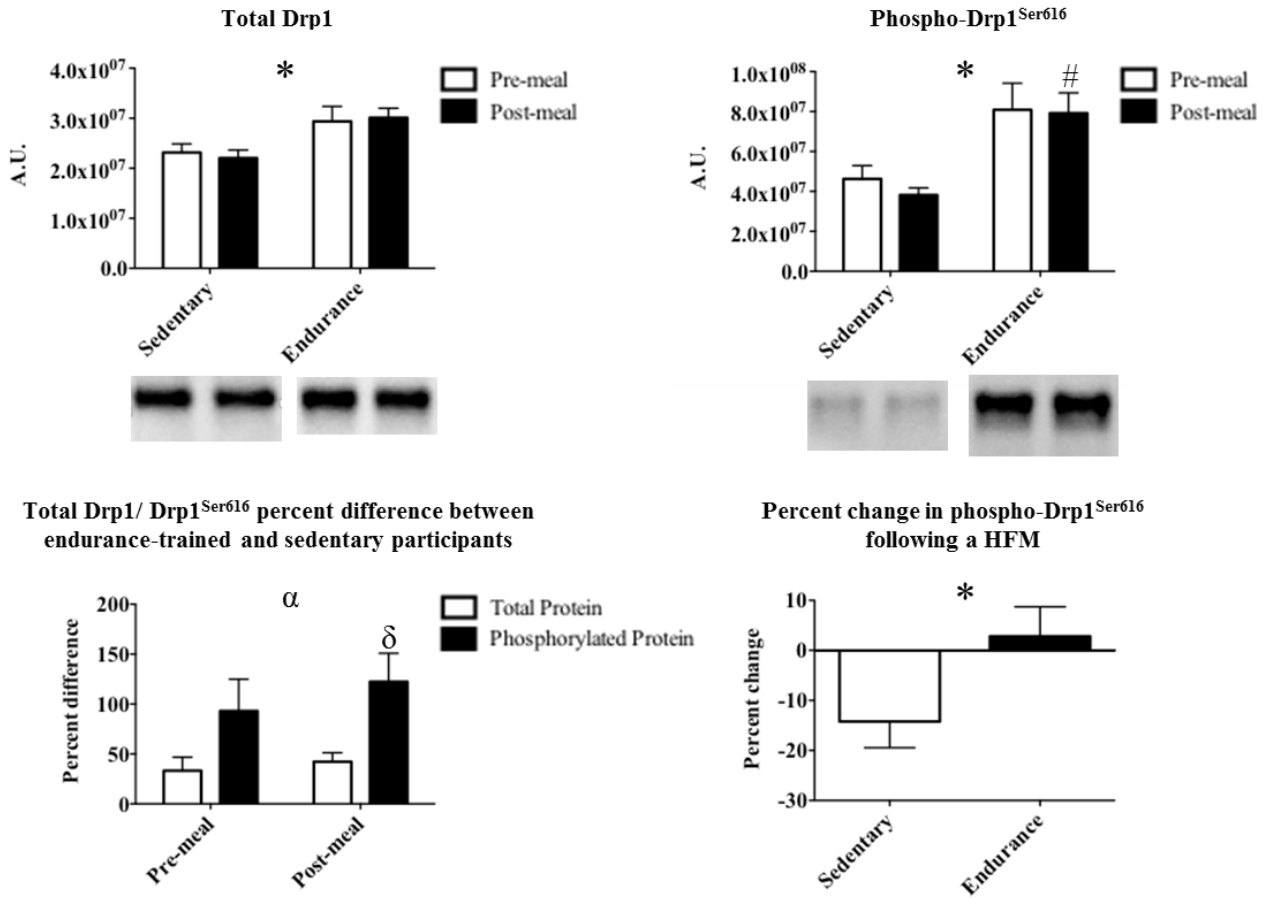


Figure 9. Assessment of skeletal muscle mitochondrial fission markers. Differences in total Drp1 and phospho-Drp1^{Ser616} between sedentary and endurance-trained groups and pre and post-meal time points. Percent differences in total Drp1 and phospho-Drp1^{Ser616} between endurance-trained and sedentary participants' at pre and post-meal time points. Percent change in phospho-Drp1^{Ser616} between sedentary and endurance-trained groups' following a HFM. Representative western blots are included below their respective graphs. Data are presented as mean ± SEM. *, significant overall group difference; #, significant time-point difference between groups'; α, significant overall difference between total and phosphorylated protein; δ, significant time point difference between total and phosphorylated protein.

There were significant group effects for complexes I-V and total ETC content (sum of complexes I-V) in favor of greater expression in skeletal muscle of endurance-trained participants'. Complex II and IV expression decreased significantly following a HFM. Reductions in complexes I ($p = 0.078$) and total ETC ($p = 0.051$) did not achieve statistical significance. Complexes I, II, III and V, and total ETC content were significantly greater in endurance-trained skeletal muscle pre and post-meal. Complex IV was significantly greater post-meal, but only trended higher pre-meal ($p = 0.069$). Total ETC content and individual complexes I, II, III and V were not significantly altered by a HFM within groups'. Complex IV was significantly reduced following a HFM in sedentary participants' only (figure 10).

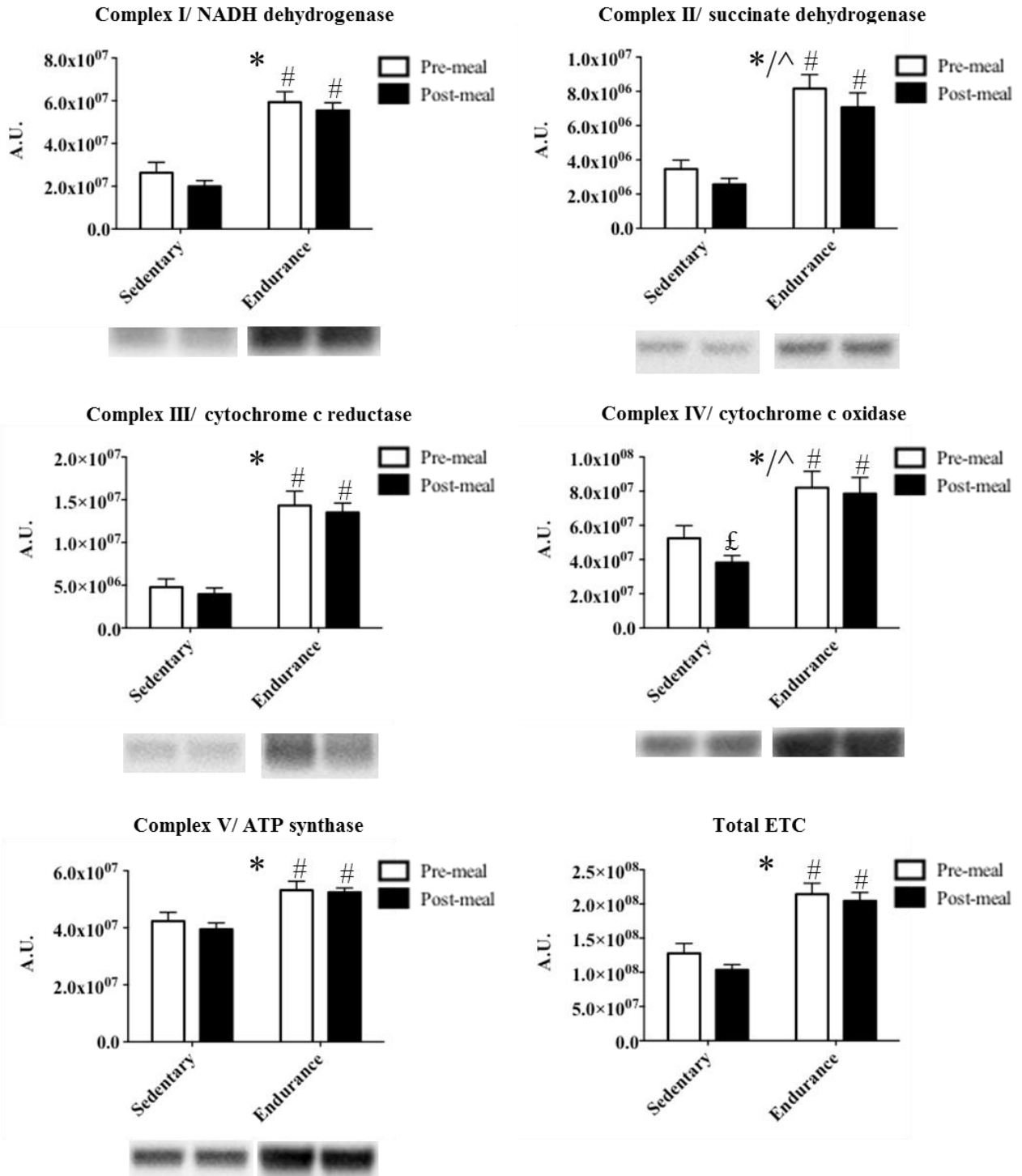


Figure 10. Assessment of changes in skeletal muscle ETC complexes and total ETC complex content. Differences in complex I/ NADH dehydrogenase, complex II/ succinate dehydrogenase, complex III/ cytochrome c reductase, complex IV/ cytochrome c oxidase, complex V/ ATP

synthase and total ETC complex content between sedentary and endurance-trained participants', at pre and post-meal time points. Representative western blots are included below their respective graphs. Data are presented as mean \pm SEM. *, significant overall group difference; #, significant time-point difference between groups'; £ significant within group time-point difference; ^, significant meal effect.

Metabolic markers. Pyruvate dehydrogenase (PDH) activity was significantly greater overall in endurance-trained skeletal muscle. PDH activity was not significantly different between groups' pre-meal, but was significantly greater in the endurance-trained group post-meal. Analysis of metabolic flexibility in skeletal muscle homogenate found no significant group effect, but did discover a significant meal effect representing an increase in metabolic flexibility post-HFM. The significant meal effect appeared to be largely due to an increase in mean metabolic flexibility post-meal in endurance-trained skeletal muscle compared to pre-meal metabolic flexibility, which was not significant, but represented a trend ($p = 0.063$) (figure 11).

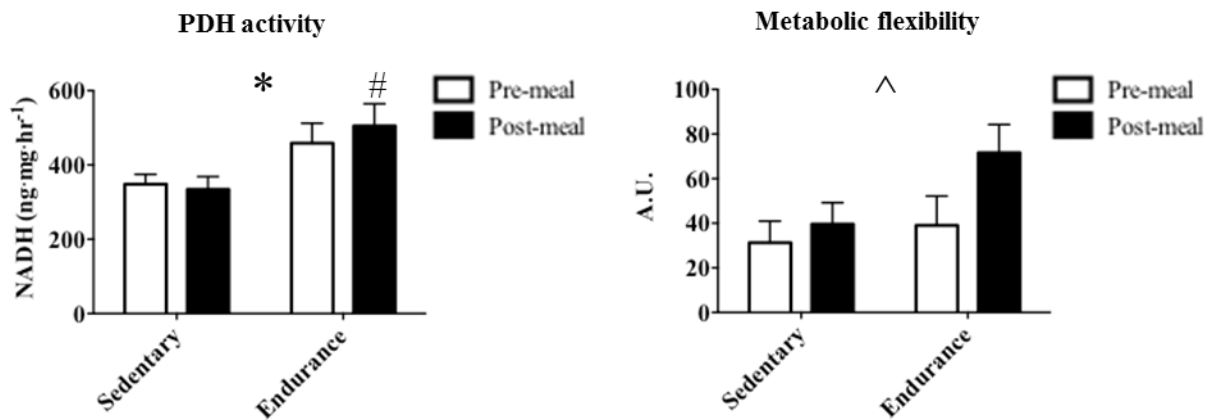


Figure 11. Assessment of markers of skeletal muscle metabolic function. Differences in PDH activity and metabolic flexibility between sedentary and endurance-trained participants'. Data are presented as mean \pm SEM. *, significant overall group difference; #, significant time-point difference between groups'; ^, significant meal effect.

Multivariate analysis. Analysis of associations between protein expression data showed that key mitophagy markers, phospho-PINK1^{Thr257} and phospho-Parkin^{Ser65} shared a significant positive correlation at pre and post-meal time points, supporting a relationship in skeletal muscle (figure 12). Both phospho-PINK1^{Thr257} and phospho-Parkin^{Ser65} showed a significant and positive relationship with phospho-Drp1^{Ser616}, suggesting cooperation between mitophagy and mitochondrial fission (figure 12). Furthermore, multivariate analysis revealed a significant positive correlation between total ETC content and phospho-PINK1^{Thr257}, phospho-Parkin^{Ser65} and phospho-Drp1^{Ser616} suggesting a possible relationship between oxidative phosphorylation capacity and mitophagy (figure 12). Oxidative capacity, determined via VO_{2max} was shown to share a significant positive relationship with total ETC content, as well as phospho-PINK1^{Thr257}, phospho-Parkin^{Ser65} and phospho-Drp1^{Ser616}, indicating a potential relationship between whole body oxidative capacity and skeletal muscle mitophagy activity (figure 13). No associations were found between skeletal muscle metabolic flexibility and markers of autophagy or mitophagy in skeletal muscle.

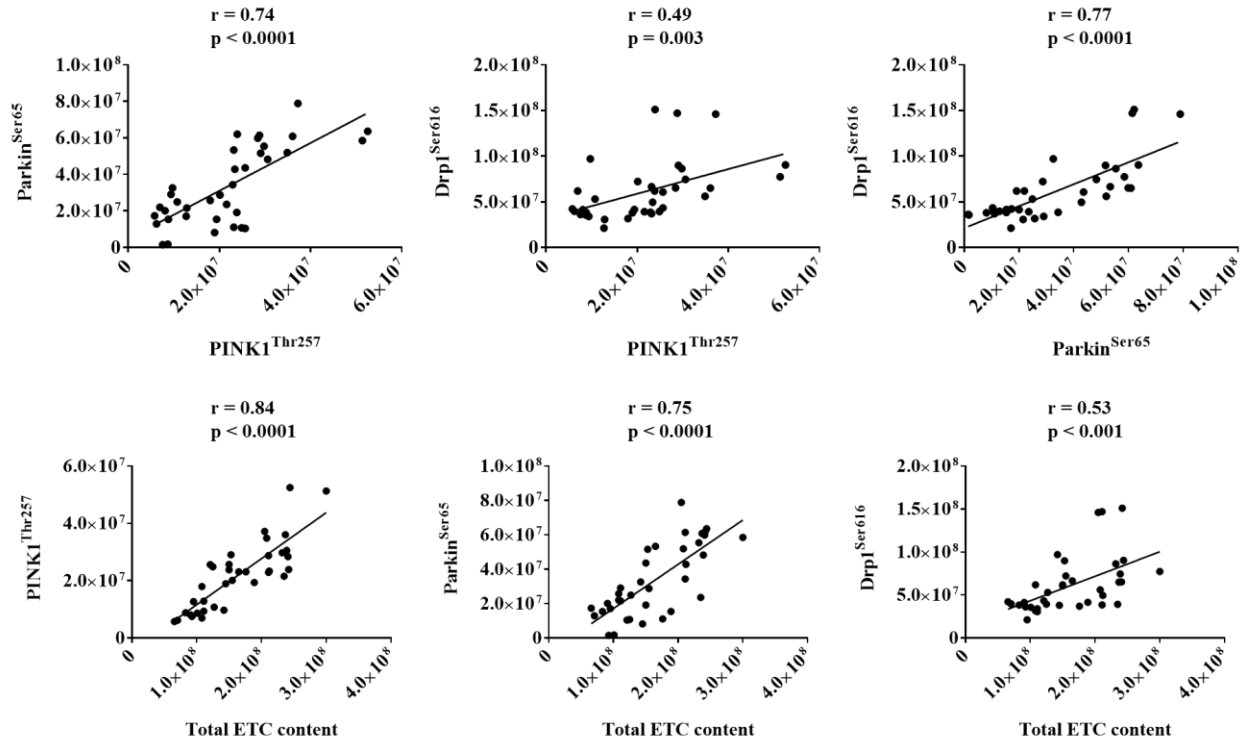


Figure 12. Multivariate analysis of protein expression for mitophagy markers and total ETC content. Statistical analysis of correlations between the protein expression of phospho-PINK1^{Thr257} and phospho-Parkin^{Ser65}, phospho-PINK1^{Thr257} and phospho-Drp1^{Ser616}, phospho-Parkin^{Ser65} and phospho-Drp1^{Ser616}, total ETC content and phospho-PINK1^{Thr257}, total ETC content and phospho-Parkin^{Ser65}, and total ETC content and phospho-Drp1^{Ser616}.

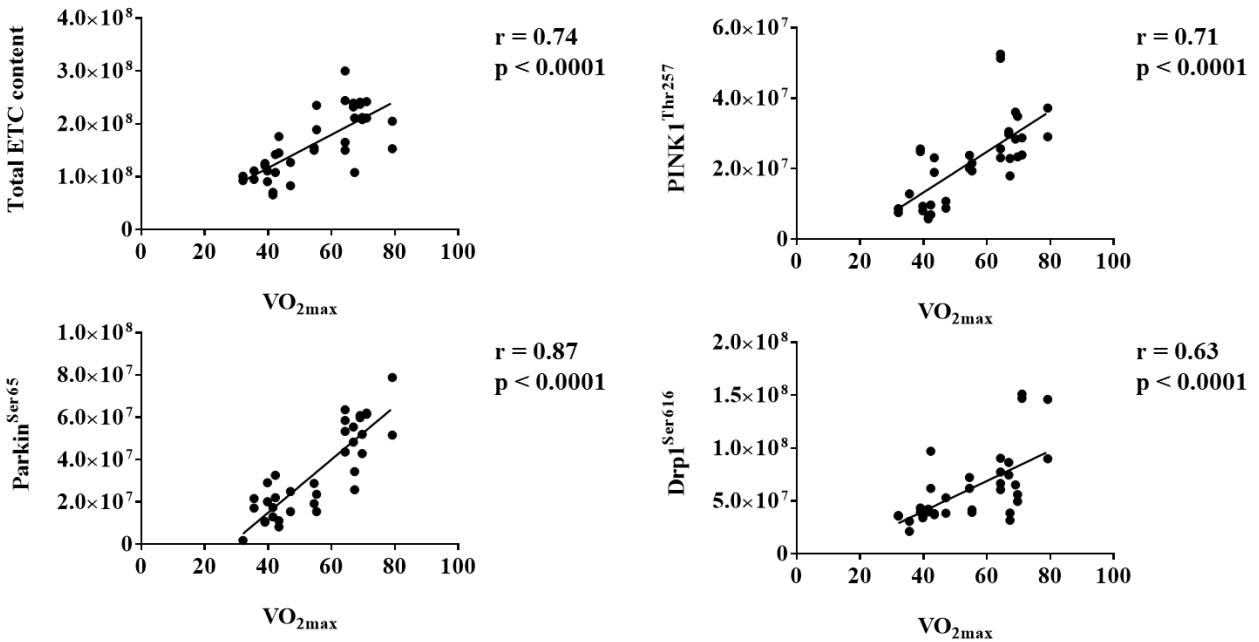


Figure 13. Multivariate analysis of VO_{2max} and protein expression of total ETC content and mitophagy markers. Statistical analysis of correlations between VO_{2max} ($ml \cdot kg \cdot min^{-1}$) and protein expression of total ETC, phospho-PINK1^{Thr257}, phospho-Parkin^{Ser65} and phospho-Drp1^{Ser616}, respectively.

DISCUSSION

The current study aimed to determine whether markers of skeletal muscle autophagy and mitophagy were different between sedentary and endurance-trained individuals' following an overnight fast, whether a single HFM influenced skeletal muscle autophagy and mitophagy differentially between sedentary and endurance-trained males', and if differences were associated with enhanced metabolic function. The primary findings of the study revealed that endurance-trained individuals' do not demonstrate significantly different skeletal muscle autophagy activity following a 12h overnight fast, or in response to a HFM compared to sedentary controls. Despite

evidence of similar autophagic activity between groups', markers of mitophagy were significantly elevated in endurance-trained males' when fasted and following a HFM relative to sedentary males'. Skeletal muscle metabolic flexibility was not significantly different between groups' when fasted or following a HFM, but was significantly increased in response to a HFM, independent of training status.

The similarities in markers of autophagy between groups' suggest that skeletal muscle adaptations to endurance exercise do not result in altered autophagy markers following an overnight fast. Acute exercise bouts are known to stimulate autophagy in skeletal muscle of trained (Jamart et al., 2012a and 2012b) and untrained humans (Moller et al., 2015), but until now it was unclear whether adaptation to endurance exercise training would cause chronically elevated skeletal muscle autophagy in humans. Lira et al., (2013) showed increased markers of basal autophagy activity in murine skeletal muscle following four weeks of endurance training, which were not supported by data from the current study. However, given the complexity of autophagy regulation and the limitations in interpreting LC3 and p62 markers, further studies are required to confirm the current findings. In addition to training status, skeletal muscle autophagy markers were also unaffected by a single HFM. Endurance-trained participants' exhibited significantly greater expression of two key mitophagy activators, phospho-PINK1^{Thr257} and phospho-Parkin^{Ser65}, compared to sedentary individuals', despite no discernable differences in general autophagy activity. The greater content of mitophagy markers during fasting and following a HFM may suggest that mitophagy activity is chronically elevated in endurance-trained skeletal muscle, and not the by-product of nutrient availability or a particular metabolic state.

The current study is the first to utilize the phosphorylation status of PINK1 and Parkin as a method for the determination of mitophagy activity in human skeletal muscle. In response to mitochondrial membrane depolarization, PINK1 autophosphorylates at several residues, including Thr257, promoting protein activation (Kondapalli et al., 2012). The phosphorylation of residue Thr257 promotes PINK1 kinase activity, leading to the phosphorylation of Parkin at Ser65, and its subsequent translocation to the outer mitochondrial membrane, and initiation of its E3 ligase activity (Kondapalli et al., 2012; Kane et al., 2014). The significant positive correlation between phospho-PINK1^{Thr257} and phospho-Parkin^{Ser65} also supports the existence of their relationship for the first time in human skeletal muscle. Parkin is degraded in the lysosome during mitophagy (Cai et al., 2012), thus the greater expression of total Parkin in sedentary skeletal muscle may represent an accumulation of Parkin in sedentary skeletal muscle stemming from a slower rate of mitophagy compared to the endurance-trained group. The pathways of mitophagy are closely aligned with those of mitochondrial dynamics. Downregulation of mitochondrial fission protein, Drp1, for example leads to elongation of the mitochondrial network and inhibition of mitophagy (Twig et al., 2008). Mitochondrial fission may therefore be a prerequisite for mitophagy. Greater Drp1 activity in endurance-trained skeletal muscle, determined by a higher phospho-Drp1^{Ser616} expression, may reflect a pro-fission state that is primed for mitophagy. Indeed, the significant positive correlation between phospho-Drp1^{Ser616} and both phospho-PINK1^{Thr257} and phospho-Parkin^{Ser65} may be evidence of such a relationship. Whether the increased expression of Mfn2 in endurance-trained skeletal muscle can be considered a counter weight to elevated fission markers is unclear in the present study.

Increased mitophagy may be expected to reduce Mfn1 and Mfn2, which become targets of Parkin-mediated degradation following mitochondrial depolarization, an event required for the activation of PINK1 and subsequently Parkin (Poole et al., 2010; Glauser et al., 2011). Interestingly, Mfn2 is a substrate of PINK1, which is capable of phosphorylating Mfn2 on at least two residues (Chen and Dorn, 2013). Mfn2 is localized to the outer mitochondrial membrane (OMM) where it functions as a receptor for Parkin following PINK1-mediated phosphorylation (Chen and Dorn, 2013). The docking of Parkin to Mfn2 at the OMM initiates the ubiquitination of mitochondrial proteins that label the organelle for mitophagy (Chen and Dorn, 2013). Elevated Mfn2 content, as the present study showed in endurance-trained skeletal muscle, may then be required during periods of enhanced mitophagic activity, again, as reflected in endurance-trained skeletal muscle. These data may provide further evidence of elevated skeletal muscle mitophagy in endurance-trained participants’.

The relevance of elevated mitophagy in endurance-trained skeletal muscle has not previously been defined, but data from the present study suggest it may be related to oxidative capacity. The significant positive correlations between OXPHOS proteins and mitophagy markers shown in the current study support a relationship between OXPHOS capacity and mitophagy activity. Furthermore, the significant positive correlations between VO_{2max} and mitophagy markers may suggest that whole body oxidative capacity is a factor in skeletal muscle mitophagy activity. Previous studies have indicated a similar relationship. Lira et al., (2013) reported elevated mitophagy markers in oxidative muscle fibers, compared to glycolytic fibers, as well as in untrained skeletal muscle from Pgc-1a overexpressor mice. Melser et al., (2013) used media to manipulate the metabolic states of human primary skeletal muscle toward states of either high or

low oxidative phosphorylation (OXPHOS). Primary skeletal muscle cells in a high OXPHOS status were more efficient, produced more reactive oxygen species, and increased basal mitophagy relative to cells in a low OXPHOS status. The current study did not determine whether OXPHOS activity was different between groups', but an abundance of ETC protein in endurance-trained skeletal muscle support an increased OXPHOS capacity.

Skeletal muscle metabolic flexibility assesses a tissue's ability to switch between glucose and fatty acid oxidation, which is relevant in the context of insulin resistance and T2D, which are associated with impaired fasting lipid oxidation (Kelley and Simoneau, 1994; Blaak et al., 2001) and excess lipid accumulation in skeletal muscle (Ellis et al., 2000; Itani et al., 2005). Findings from the current study contradict those of Dube et al., (2015) who reported increased metabolic flexibility in endurance-trained participants' compared to untrained controls. Participant characteristics were similar between studies, although Dube and colleagues (2015) used a lipid infusion protocol, which may alter metabolic outcomes through bypassing the gastrointestinal system. Dube et al., (2015) concluded that greater metabolic flexibility in endurance-trained individuals' was the result of enhanced mitochondrial performance. Mitophagy is necessary for mitochondrial quality control and optimal mitochondrial performance, which is compromised in skeletal muscle of individuals' with metabolic disease who experience a range of mitochondrial defects (Kelley et al., 2002; Befroy et al., 2007; van de Weijer; Vijgen et al., 2013; Kristensen et al., 2014; Hernandez-Alvarez et al., 2010; Ritov et al., 2010; Wijngaarden et al., 2013; Mootha et al., 2003; Patti et al., 2003; Petersen et al., 2004; Morino et al., 2005; Boushel et al., 2007; Rabol et al., 2009). Increased OXPHOS proteins combined with elevated mitophagy shown in the current study may imply that endurance-trained individuals' exhibit an enhanced mitochondrial quality control. These factors

were not associated with improved metabolic flexibility in endurance-trained males however. Increases in oxidative capacity, mitochondrial OXPHOS proteins and mitophagy activity may not then confer advantages to skeletal muscle substrate metabolism compared to sedentary individuals. Alternatively, sedentary behaviors in the absence of pre-existing metabolic disorders may not impair skeletal muscle substrate metabolism.

To the authors' knowledge this is the first study to investigate differences in skeletal muscle autophagy and mitophagy between sedentary and endurance-trained humans. As such, comparative literature is limited. Previously, a downregulation of mitophagy markers has been shown in skeletal muscle of inactive frail older women compared to active older women following an overnight fast (Drummond et al., 2014). While the downregulation of mitophagy markers in the inactive group may concur with those of the current study, different population were examined. Chavez et al., (2010) noted a 33% reduction in mitochondrial membrane potential in lean, healthy males following an 8h lipid infusion, which might suggest the activation of mitophagy via membrane potential-sensitive PINK1. However, autophagy and mitophagy protein expression was not measured. Regardless, it is not clear that findings between an 8h lipid infusion and a HFM would be translatable. Lira et al., (2013) concluded that four weeks of endurance training increased basal autophagy activity and mitophagy protein expression, specifically, increased Bnip3 expression. Without additional indicators of mitophagy activity it was not possible to determine whether increased basal autophagy is synonymous with increased mitophagy (Lira et al., 2013). Data from the current study would suggest not, although it is not clear why. Increases in the mitophagy marker, Bnip3, were dependent on an enhanced oxidative phenotype (Lira et al., 2013). Thus, predominantly oxidative fibers, such as the mouse soleus muscle used by Lira and colleagues

(2013), did not increase Bnip3 in response to exercise, while mixed fiber type plantaris muscle did. The current study took biopsies from the vastus lateralis, a mixed fiber muscle (Staron et al., 2000). If the results of Lira et al., (2013) are translatable to humans then it may be expected that the vastus lateralis would demonstrate positive mitophagy responses to endurance training, which was the case in the current study.

The study experienced several limitations. Firstly, the use of LC3 to determine autophagic flux, particular under static conditions such as the current study presents several challenges due to the transient nature of LC3, which is degraded by the autolysosome (Klionsky et al., 2012). Furthermore, the association between p62 expression and autophagic flux has been challenged (Sahani, Itakua and Mizushima, 2014). These data should therefore be interpreted with caution. The cross-sectional study design used may not be ideal for assessing the impact of endurance training on skeletal muscle. The current study was unable to control for different training patterns and recruited endurance-trained runners across a wide spectrum of endurance running events. These factors may have increased variation, which combined with the relative low n value may have increased the risk of a type II error. The current study assessed habitual fat intake via food diaries to exclude individuals that may have be acclimated to excess fat consumption. Dietary controls were no in place to control eating habits beyond 12 hours prior to the first biopsy, which may have influenced our data. The introduction of dietary guidelines prior to testing or a controlled feeding period may reduce error in future studies.

In conclusion, skeletal muscle of endurance-trained males exhibits elevated markers of mitophagy activity during fasting and following a HFM compared to sedentary males, without a concurrent increase in markers of autophagy. The mechanism of enhanced mitophagy in response to endurance training is not apparent, but may relate to substantial differences in skeletal muscle oxidative phenotype and oxidative capacity of the participants'. Consumption of a HFM was not sufficient to stimulate changes in autophagy or mitophagy in either group, although as sedentary controls' were considered to be in good metabolic health they may be capable of compensating against such metabolic insults. The similar metabolic flexibility between groups' might support such a compensatory ability, although the data were highly variable. Future studies should aim to replicate the current work, expanding the available literature, while investigating populations with obesity, insulin resistance and/ or type II diabetes to develop our understanding of the association between metabolic health and skeletal muscle autophagy and mitophagy. The utilization of a wider array of methodological techniques may aid future studies when interpretation autophagy marker data. Finally, employing longitudinal training studies' in place of a cross-sectional design may be more informative.

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Wijngaarden, M. A., van der Zon, G. C., van Dijk, K. W., Pijl, H., & Guigas, B. (2013). Effects of prolonged fasting on AMPK signaling, gene expression, and mitochondrial respiratory chain content in skeletal muscle from lean and obese individuals. *Am J Physiol Endocrinol Metab*, 304(9), E1012-1021. doi: 10.1152/ajpendo.00008.2013

Zimmet, P., Alberti, K. G., & Shaw, J. (2001). Global and societal implications of the diabetes epidemic. *Nature*, 414(6865), 782-787. doi: 10.1038/414782a

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Our first study found markers of reduced skeletal muscle autophagy in response to a HFD. We also showed that autophagy may be reduced in response to a single HFM-independent of the HFD. Comparison of the autophagy and mitophagy responses to a HFM between our studies produce conflicting results. Direct comparisons are confounded though by the two-week controlled diet employed in the 5-day HFD study. In addition, interpretations' of the data were problematic due to methodological limitations. We cannot therefore confirm that autophagy was reduced following either a HFM or a HFD. The current study showed minor evidence of significantly altered mitophagy activity. Mitophagy may be reduced in response to a high-fat meal independently of the HFD based on reductions in phosphorylated Parkin. However, this was not supported by similar changes in PINK1 or Drp1. We tested the hypothesis that a 5-day HFD would reduce skeletal muscle autophagy and mitophagy, and while our findings do not support this, consumption of a HFD may still be detrimental. Future studies should consider using longer periods, which may better reflect habitual high-fat intake. Interpreting the responses of sedentary individuals to a HFD may also be aided by comparing their responses to those of endurance-trained individuals' consuming the same HFD. It would also be an extension of our second study to assess whether adaptations to endurance training protect against excess fat intake

The major findings from our second study are that markers of skeletal muscle mitophagy are elevated in endurance-trained male runners' in the absence of any discernable increase in autophagy. The reason for this is not clear, but may be due to limitations in accurately measuring

autophagy protein markers. Increases in mitophagy markers were correlated with maximal oxygen consumption and ETC proteins. It is possible therefore that mitophagy activity is regulated by oxidative capacity and/ or the size of the mitochondrial population. Further studies are required to confirm this. Skeletal muscle metabolic flexibility was not significantly different between groups' following an overnight fast or in response to a HFM, nor were any associations found between metabolic flexibility and mitophagy markers, maximal oxygen consumption or ETC content. Metabolic flexibility may therefore be governed by other factors not closely associated with endurance exercise adaptations. Alternatively, young, sedentary, healthy, non-obese males may possess sufficient plasticity in skeletal muscle metabolism to compensate for fasting and high-fat metabolic stresses. A detailed comparative analysis of mitochondrial respiration and substrate oxidation between endurance-trained and sedentary individuals' may help distinguish the relationship between metabolic flexibility and mitochondrial quality. The data contained several statistical trends and instances of substantially different means that were non-significant due to high variation. In future, longitudinal training studies in previously sedentary individuals' may provide a more tightly controlled environment for assessing the relationship between endurance training and autophagy/ mitophagy, as well as any association to metabolic flexibility and insulin sensitivity.

As already discussed, the use of young, sedentary, healthy and non-obese participants may conceal detrimental effects of high-fat feeding-induced metabolic stress. Alternatively, such stress may not cause defects in skeletal muscle autophagy and mitophagy activity. It may be necessary to develop autophagy and mitophagy profiles for elderly individuals' and those with metabolic disease to confirm the existence of disruptions in autophagy and mitophagy, and whether a relationship exists

with metabolic function. The major limitation of the current study was the inability to accurately interpret markers of autophagy to determine whether changes occurred. Future studies may resolve this through the application of assay techniques using primary human skeletal muscle cells or muscle fibers. This would provide a means to assess autophagy in real-time, thus removing the limitations imposed by static measures.

APPENDICES

Appendix A: Approved Institutional Review Board Research Protocol



Institutional Review Board
Research Protocol

Once complete, upload this form as a Word document to the IRB Protocol Management System:
<https://secure.research.vt.edu/irb>

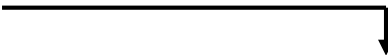
Section 1: General Information

1.1 DO ANY OF THE INVESTIGATORS OF THIS PROJECT HAVE A REPORTABLE CONFLICT OF INTEREST?

<http://www.irb.vt.edu/pages/researchers.htm#conflict>

- No
- Yes, explain:

1.2 WILL THIS RESEARCH INVOLVE COLLABORATION WITH ANOTHER INSTITUTION?

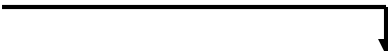
- No, go to question 1.3
- Yes, answer questions within table 

IF YES
<p>Provide the name of the institution [for institutions located overseas, please also provide name of country]:</p>
<p>Indicate the status of this research project with the other institution's IRB:</p> <p><input type="checkbox"/> Pending approval</p>

<input type="checkbox"/> Approved <input type="checkbox"/> Other institution does not have a human subject protections review board <input type="checkbox"/> Other, explain:
<p>Will the collaborating institution(s) be engaged in the research?</p> <p>http://www.hhs.gov/ohrp/policy/engage08.html</p> <input type="checkbox"/> No <input type="checkbox"/> Yes
<p>Will Virginia Tech's IRB review all human subject research activities involved with this project?</p> <input type="checkbox"/> No, provide the name of the primary institution: <input type="checkbox"/> Yes <p><i>Note: primary institution = primary recipient of the grant or main coordinating center</i></p>

1.3 IS THIS RESEARCH FUNDED?

No, go to question 1.4

Yes, answer questions within table 

IF YES
<p>Provide the name of the sponsor [if NIH, specify department]:</p>
<p>Is this project receiving federal funds?</p> <input type="checkbox"/> No <input type="checkbox"/> Yes

If yes,

Does the grant application, OSP proposal, or “statement of work” related to this project include activities involving human subjects that are not covered within this IRB application?

- No, all human subject activities are covered in this IRB application
- Yes, however these activities will be covered in future VT IRB applications, these activities include:
- Yes, however these activities have been covered in past VT IRB applications, the IRB number(s) are as follows:
- Yes, however these activities have been or will be reviewed by another institution’s IRB, the name of this institution is as follows:
- Other, explain:

Is Virginia Tech the primary awardee or the coordinating center of this grant?

- No, provide the name of the primary institution:
- Yes

1.4 DOES THIS STUDY INVOLVE CONFIDENTIAL OR PROPRIETARY INFORMATION (OTHER THAN HUMAN SUBJECT CONFIDENTIAL INFORMATION), OR INFORMATION RESTRICTED FOR NATIONAL SECURITY OR OTHER REASONS BY A U.S. GOVERNMENT AGENCY?

For example – government / industry proprietary or confidential trade secret information

- No
- Yes, describe:

1.5 DOES THIS STUDY INVOLVE SHIPPING ANY TANGIBLE ITEM, BIOLOGICAL OR SELECT AGENT OUTSIDE THE U.S.?

- No
- Yes

Section 2: Justification

2.1 DESCRIBE THE BACKGROUND, PURPOSE, AND ANTICIPATED FINDINGS OF THIS STUDY:

Obesity and diabetes are associated with an accumulation of damaged, dysfunctional mitochondria causing reduced oxidative capacity and increased reactive oxygen species production. Mitochondrial quality control is dependent on mitochondrial turnover, which is the removal and replacement of damaged, dysfunctional mitochondria with new, healthy mitochondria. Mitochondrial turnover is dependent on the coordinated interactions of mitochondrial biogenesis and autophagy and while much is known about the pathways regulating mitochondrial biogenesis, little is known about the regulation of autophagy, especially in skeletal muscle in humans. Endurance exercise is known to activate autophagy in rodent models, however whether a similar pattern occurs in humans is not currently known. We hypothesize that endurance trained individuals will display an up regulation of autophagic pathways, which will be associated with improved metabolic function in skeletal muscle when compared to sedentary individuals. Therefore, the current study will examine regulators of autophagy in skeletal muscle from endurance trained individuals compared to age and sex matched, sedentary individuals. Additionally, we will utilize a high fat meal challenge to determine whether the pathways regulating autophagy are more responsive to changes in energy status than sedentary individuals, which would confer better health outcomes. Results from this work will increase our understanding of how skeletal muscle autophagy is regulated and whether an up regulation of autophagy with endurance training is a potential mechanism of obesity and diabetes resistance.

2.2 EXPLAIN WHAT THE RESEARCH TEAM PLANS TO DO WITH THE STUDY RESULTS:

For example - publish or use for dissertation

The results of the proposed study will be published in peer reviewed publications, generate data for grant proposals, and may be used for dissertation research (graduate student listed in the personnel; other students may be added at a later date).

Section 3: Recruitment

3.1 DESCRIBE THE SUBJECT POOL, INCLUDING INCLUSION AND EXCLUSION CRITERIA AND NUMBER OF SUBJECTS:

Examples of inclusion/exclusion criteria - gender, age, health status, ethnicity

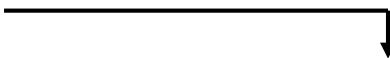
Thirty healthy, non obese men 18-45 years of age of all races and ethnic backgrounds will serve as subjects. This study will recruit two groups of individuals: one group will include 15 endurance trained individuals (>5 hours per week in running/cycling and at least two competitive endurance events in the past year) and one group will include 15 sedentary individuals (<2 days per week of physical activity/ exercise <20 min). All subjects will undergo a health screening and only those individuals with no history of cardiopulmonary disease and meeting the following criteria will be included: 1. non-smoking. 2. blood pressure <140/90mmHg. 3. total cholesterol <240 mg/dl or triglycerides <300 mg/dl. 4. fasting blood glucose <126 mg/dl. 5. BMI < 25.30 kg/m². All subjects will be weight stable (< approximately 2.5 kg) for the past 6 months and at their maximal body weight. Subjects will be excluded if they are taking any medications or supplements that could influence any of the dependent variables. Subjects will otherwise be healthy as determined from the health history. Training status and physical activity level will be determined by self-report and maximal oxygen consumption (VO₂max).

3.2 WILL EXISTING RECORDS BE USED TO IDENTIFY AND CONTACT / RECRUIT SUBJECTS?

Examples of existing records - directories, class roster, university records, educational records

No, go to question 3.3

Yes, answer questions within table



IF YES	
Are these records private or public?	
<input type="checkbox"/> Public	
<input type="checkbox"/> Private, describe the researcher's privilege to the records:	
Will student, faculty, and/or staff records or contact information be requested from the University?	
<input type="checkbox"/> No	
<input type="checkbox"/> Yes, visit the following link for further information: http://www.policies.vt.edu/index.php (policy no. 2010)	



3.3 DESCRIBE RECRUITMENT METHODS, INCLUDING HOW THE STUDY WILL BE ADVERTISED OR INTRODUCED TO SUBJECTS:

Individuals will be recruited through advertisement. We anticipate recruiting through posted fliers, emails, and newspaper advertisements. Individuals will be provided with a phone number and an email address for initial contact to inquire about the study. Subjects will either be screened by phone or via online using an online screening tool. An initial phone screening will be used to exclude individuals based on BMI, health status, and medication usage.

3.4 PROVIDE AN EXPLANATION FOR CHOOSING THIS POPULATION:

Note: the IRB must ensure that the risks and benefits of participating in a study are distributed equitably among the general population and that a specific population is not targeted because of ease of recruitment.

We are recruiting men 18-45 years of age of all races and ethnic backgrounds. We have decided to only include males in this study since it is proof of concept in a research area not previously addresses humans. As such, previous work has demonstrated that these outcomes may be impacted by female hormones and menstrual cycle. Examining these questions in females is a future direction of the project.

Section 4: Consent Process

For more information about consent process and consent forms visit the following link:
<http://www.irb.vt.edu/pages/consent.htm>

If feasible, researchers are advised and may be required to obtain signed consent from each participant unless obtaining signatures leads to an increase of risk (e.g., the only record linking the subject and the research would be the consent document and the principal risk would be potential harm resulting in a breach of confidentiality). Signed consent is typically not required for low risk questionnaires (consent is implied) unless audio/video recording or an in-person interview is involved. If researchers will not be obtaining signed consent, participants must, in most cases, be supplied with consent information in a different format (e.g., in recruitment document, at the beginning of survey instrument, read to participant over the phone, information sheet physically or verbally provided to participant).

4.1 CHECK ALL OF THE FOLLOWING THAT APPLY TO THIS STUDY'S CONSENT PROCESS:

- Verbal consent will be obtained from participants
- Written/signed consent will be obtained from participants
- Consent will be implied from the return of completed questionnaire. Note: The IRB recommends providing consent information in a recruitment document or at the beginning of the questionnaire (if the study only involves implied consent, skip to Section 5 below)
- Other, describe:

4.2 PROVIDE A GENERAL DESCRIPTION OF THE PROCESS THE RESEARCH TEAM WILL USE TO OBTAIN AND MAINTAIN INFORMED CONSENT:

Those individuals who respond to the advertisements will be contacted by phone where they will be told the general plan of the study and all specific procedures included in the study. If requested, examples of biopsy scars will be sent electronically and individuals will be given a chance to answer any questions regarding study procedures. Those still interested will be screened over the phone to determine eligibility based on BMI, health status, and medication usage. Eligible individuals will be sent a copy of the consent form via email to review prior to coming to the lab. They will then be given a chance to ask any questions either by email or when then come to the lab. Those still interested will be asked to sign the consent.

4.3 WHO, FROM THE RESEARCH TEAM, WILL BE OVERSEEING THE PROCESS AND OBTAINING CONSENT FROM SUBJECTS?

Madlyn Frisard, Ph.D. will be responsible for this and all aspects of the study.

4.4 WHERE WILL THE CONSENT PROCESS TAKE PLACE?

Human Integrative Physiology Laboratory in War Memorial Hall

4.5 DURING WHAT POINT IN THE STUDY PROCESS WILL CONSENTING OCCUR?

Note: unless waived by the IRB, participants must be consented before completing any study procedure, including screening questionnaires.

In the initial contact with the subject, the study will be explained to them and they will receive a copy of the informed consent.

4.6 IF APPLICABLE, DESCRIBE HOW THE RESEARCHERS WILL GIVE SUBJECTS AMPLE TIME TO REVIEW THE CONSENT DOCUMENT BEFORE SIGNING:

Note: typically applicable for complex studies, studies involving more than one session, or studies involving more of a risk to subjects.

Potential participants will be given a copy of the consent to take home with them to review. They will then return at a later date with the consent and this is to ensure they have had enough time to review the consent and have any questions answered.

Not applicable

Section 5: Procedures

5.1 PROVIDE A STEP-BY-STEP THOROUGH EXPLANATION OF ALL STUDY PROCEDURES EXPECTED FROM STUDY PARTICIPANTS, INCLUDING TIME COMMITMENT & LOCATION:

Participants will participate in both sessions below.

Testing Sessions will be held in War Memorial Hall

Participants will participate in all testing sessions once. The number and order of the testing sessions may be changed based on participants time and schedule.

Session One: (Approximately 3 hr)

Overnight Fast: Subjects will be asked to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food they eat or by the normal digestion process.

Medical History: Subjects will be asked to complete a medical history questionnaire, which will be used to screen for health problems or any reason an individual should be excluded from the study

Resting Blood Pressure and Heart Rate. Blood pressure measurements will be made under quiet, comfortable ambient laboratory conditions via mercury sphygmomanometry. Measurements will conform strictly to American Heart Association guidelines.

Physical activity questionnaire: Subjects will be asked to fill out a questionnaire concerning their previous physical activity level.

Catheter and Blood Draw: A small plastic tube will be inserted into the subject's arm to draw blood (approximately 3 tablespoons). The blood will be used to measure fasting glucose, insulin, lipids, and other factors that may affect a subjects health.

Body Mass and Composition. Body weight will be measured on a digital scale accurate to +0.01 kg. Height will be measured with a standard stadiometer. Percent body fat and fat-free mass will be measured in all subjects using dual-energy x-ray absorptiometry (DEXA) (Prodigy Advance, GE Healthcare).

Maximal Oxygen Consumption (VO₂ max). Maximal oxygen consumption will be measured using a standard ramp test protocol lasting 8-12 minutes. Briefly, subjects will run on a treadmill at a fixed pace with gradient increases at one minute increments until volitional exhaustion. Oxygen consumption will be measured throughout using a metabolic cart.

Take home tests: Subjects will be asked to record all of the food they eat during a 4 day period.

Session 2: (Approximately 5 hours)

Overnight Fast: Subjects will be asked to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food they eat or by the normal digestion process.

Infection/Inflammation Questionnaire: Subjects will be asked to complete a questionnaire about any recent illnesses or infections that they may have had in the past month.

Skeletal Muscle Biopsy. A vastus lateralis muscle biopsy will be performed using the technique of Bergström to determine whether autophagy is altered in skeletal muscle from endurance trained individuals compared to sedentary individuals. After cleansing the skin with povidone-iodine solution, the skin, adipose tissue and skeletal muscle fascia will be anesthetized using xylocaine. The skin will be incised (0.75cm) with a #11 scalpel. The

fascia fibers will be separated with the blunt edge of the scalpel and the Bergström needle (4mm) inserted into the vastus lateralis. After suction is applied, approximately 500-600 mg of muscle tissue will be removed. Pressure will be applied and the skin will be closed with sterile tape. There is a slight risk of pain, infection, scarring, bleeding, and loss of sensation around the biopsy site. Subjects will be asked to return to the lab within 5 days to have the biopsy site examined in order to ensure proper healing.

Catheter and Blood Draw: A small plastic tube will be inserted into the subject's arm to draw blood (approximately 3 tablespoons). Blood will be collected before the meal challenge and at 1, 2, 3, and 4 hours after the meal. The catheters will remain in the arm throughout the entire test.

Meal Challenge: Subjects will be asked to eat a test meal consisting of two breakfast sandwiches (e.g., egg and sausage). Blood will be collected before and at each of the subsequent 4 hours. A muscle biopsy will be performed before and approximately 4 hours after the meal.

5.2 DESCRIBE HOW DATA WILL BE COLLECTED AND RECORDED:

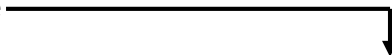
Study data will be collected on data sheets (see attached) and manually entered into a database (excel format).

5.3 DOES THE PROJECT INVOLVE ONLINE RESEARCH ACTIVITIES (INCLUDES ENROLLMENT, RECRUITMENT, SURVEYS)?

View the "Policy for Online Research Data Collection Activities Involving Human Subjects" at <http://www.irb.vt.edu/documents/onlinepolicy.pdf>

No, go to question 6.1

Yes, answer questions within table



IF YES

Identify the service / program that will be used:

www.survey.vt.edu, go to question 6.1

Blackboard, go to question 6.1

Center for Survey Research, go to question 6.1

Other

IF OTHER:

Name of service / program:

URL:

This service is...

Included on the list found at:

<http://www.irb.vt.edu/pages/validated.htm>

Approved by VT IT Security

An external service with proper SSL or similar encryption (https://) on the login (if applicable) and all other data collection pages.

None of the above (note: only permissible if this is a collaborative project in which VT individuals are only responsible for data analysis, consulting, or recruitment)

Section 6: Risks and Benefits

6.1 WHAT ARE THE POTENTIAL RISKS (E.G., EMOTIONAL, PHYSICAL, SOCIAL, LEGAL, ECONOMIC, OR DIGNITY) TO STUDY PARTICIPANTS?

Blood Draw: The P.I. or a trained technician will perform all blood draws. Aseptic conditions will be followed during all of the procedures. Universal precautions will be taken in collection and handling of all blood samples. Subjects will be told that their blood will be analyzed for presence of HIV if an experimenter is exposed to their blood.

Maximal Oxygen Consumption: American College of Sports Medicine (ACSM) states that the risk of a complications requiring hospitalization, acute myocardial infarction, and cardiac death during or immediately after a maximal exercise test is <0.20, 0.04, and 0.01%, respectively or approximately 20 hospitalization, 4 acute myocardial infarctions, and 1 cardiac death per 10,000 tests (ACSM Guidelines, 2014). As most of the studies that contribute to these statistics have involved testing of individuals at elevated risk of disease, it is likely that testing of the young, well-screened subjects in our study are of

even lower risk. The subjects we intend to use are at very low risk because of their young age and screening to eliminate those with elevated risk. Subjects selected will be in the "low risk" stratification according to ACSM guidelines (2014), i.e. wo/men less than 40 years of age, asymptomatic of disease, and positive for no other risk factors except a sedentary lifestyle. Subjects will be monitored throughout the exercise test by the researchers for signs and symptoms of cardiovascular problems (e.g. abnormal gait, pale, shortness of breath, angina). Fatigue, muscle soreness, and muscle strains could result from the exercise tests.

HIV/ Hepatitis B/ Hepatitis C: In the event a researcher or other staff person is improperly exposed to your blood, your blood will be tested for the presence of HIV, the Hepatitis B Virus, and the Hepatitis C Virus. There will not be any cost to you for this test. The research team will follow proper procedures for testing and reporting as outlined by Virginia State Law, which includes sending the sample to a certified laboratory. Please note that, should your blood require testing, you will be informed of your test results and provided with the opportunity to receive appropriate and timely counseling. In addition, your results will be sent to the local health department.

DEXA Scan: The amount of radiation that subjects will receive in the DEXA exam is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount subjects will receive is equal to 1/20 of a chest x-ray. The more radiation an individual receives over the course of their lifetime, the more likely that individual's risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks, however the exact increase in such risk is not known.

Muscle Biopsies: For all the muscle biopsies, there may be slight discomfort and burning when the local anesthetic is injected prior to the biopsy, but the subject should not feel significant discomfort during the actual biopsy procedure. Bruising in the area of the biopsy for 1-2 weeks will likely occur, but local pressure and ice are applied to the site immediately to limit this potential and its accompanying tenderness. There is a slight risk of infection at the biopsy site, however subjects will be required to return to the lab within 5 days following the biopsy to have the site checked to ensure proper healing. There is a small risk that the subject will become lightheaded, dizzy, or anxious before, during or after the procedure. There is also a small risk of fainting. If he/she feels dizzy, lightheaded or they feel faint, they should sit down or lie down immediately to avoid falling. These reactions are usually temporary and resolve within a short time after sitting or lying down. If these feeling do not go away soon after sitting or lying down, they will be instructed to call 911 or have someone take them to the nearest emergency room. We had one individual faint and hit their head after leaving the laboratory. This required a trip to the emergency room and several stitches. However, this is the only occurrence of this type out of ~350 biopsies performed in our laboratory.

Subjects will be shown pictures of a typical biopsy scar. It will also be explained that the pictures are just one example of scarring and that individuals will scar differently.

It is not possible to identify all potential risks. However, the study doctors and staff will take all possible safeguards to minimize any known and potential risks to their well being. All of the procedures are well established and used routinely in the investigators laboratory.

6.2 EXPLAIN THE STUDY'S EFFORTS TO REDUCE POTENTIAL RISKS TO SUBJECTS:

Blood Draw: The P.I. or a trained technician will perform all blood draws. Aseptic conditions will be followed during all of the procedures. Universal precautions will be taken in collection and handling of all blood samples. Subjects will be told that their blood will be analyzed for presence of HIV if an experimenter is exposed to their blood.

Muscle Biopsy: The muscle biopsies will be performed by a trained investigator or technician under the supervision of Dr. Jose Rivero. The possible risk involved with the biopsies are minimized by having trained individuals use aseptic techniques. In addition, subjects will be asked to return to lab within 5 days following the biopsy in order to ensure proper healing.

6.3 WHAT ARE THE DIRECT OR INDIRECT ANTICIPATED BENEFITS TO STUDY PARTICIPANTS AND/OR SOCIETY?

There are no direct benefits of participation. Individuals will receive health information including body composition, blood pressure, fasting glucose, insulin, and lipids, fitness level (maximal oxygen consumption/ VO2 max), and dietary intake. The individual will be compensated \$100 for participating in the study.

Section 7: Full Board Assessment


7.1 DOES THE RESEARCH INVOLVE MICROWAVES/X-RAYS, OR GENERAL ANESTHESIA OR SEDATION?

No

Yes

7.2 DO RESEARCH ACTIVITIES INVOLVE PRISONERS, PREGNANT WOMEN, FETUSES, HUMAN IN VITRO FERTILIZATION, OR MENTALLY DISABLED PERSONS?

No, go to question 7.3

Yes, answer questions within table 

IF YES	
This research involves:	
<input type="checkbox"/> Prisoners	
<input type="checkbox"/> Pregnant women	<input type="checkbox"/> Fetuses <input type="checkbox"/> Human in vitro fertilization
<input type="checkbox"/> Mentally disabled persons	

7.3 DOES THIS STUDY INVOLVE MORE THAN MINIMAL RISK TO STUDY PARTICIPANTS?

Minimal risk means that the probability and magnitude of harm or discomfort anticipated in the research are not greater in and of themselves than those ordinarily encountered in daily activities or during the performance of routine physical or psychological examinations or tests. Examples of research involving greater than minimal risk include collecting data about abuse or illegal activities. Note: if the project qualifies for Exempt review (<http://www.irb.vt.edu/pages/categories.htm>), it will not need to go to the Full Board.

No

Yes

IF YOU ANSWERED “YES” TO **ANY ONE** OF THE ABOVE QUESTIONS, 7.1, 7.2, OR 7.3, THE BOARD MAY REVIEW THE PROJECT’S APPLICATION MATERIALS AT ITS MONTHLY MEETING. VIEW THE FOLLOWING LINK FOR DEADLINES AND ADDITIONAL INFORMATION: <http://www.irb.vt.edu/pages/deadlines.htm>

Section 8: Confidentiality / Anonymity

For more information about confidentiality and anonymity visit the following link:
<http://www.irb.vt.edu/pages/confidentiality.htm>


8.1 WILL PERSONALLY IDENTIFYING STUDY RESULTS OR DATA BE RELEASED TO ANYONE OUTSIDE OF THE RESEARCH TEAM?

For example – to the funding agency or outside data analyst, or participants identified in publications with individual consent

- No**
- Yes**, to whom will identifying data be released?

8.2 WILL ANY STUDY FILES CONTAIN PARTICIPANT IDENTIFYING INFORMATION (E.G., NAME, CONTACT INFORMATION, VIDEO/AUDIO RECORDINGS)?

Note: if collecting signatures on a consent form, select “Yes.”

- No**, go to question 8.3
- Yes**, answer questions within table 

IF YES
Describe if/how the study will utilize study codes: Each subject will receive an I.D. that will contain no identifiable information.
If applicable, where will the key [i.e., linked code and identifying information document (for instance, John Doe = study ID 001)] be stored and who will have access? Study codes and identifying information will be kept in a locked cabinet in the office of the P.I. Dr Frisard.
<i>Note: the key should be stored separately from subjects’ completed data documents and accessibility should be limited.</i>
<i>The IRB strongly suggests and may require that all data documents (e.g., questionnaire responses, interview responses, etc.) do not include or request identifying information (e.g., name, contact information, etc.) from participants. If you need to link subjects’ identifying information to subjects’ data documents, use a study ID/code on all data documents.</i>

8.3 WHERE WILL DATA BE STORED?

Examples of data - questionnaire, interview responses, downloaded online survey data, observation recordings, biological samples

They will be stored in a locked cabinet in the Integrative Physiology Laboratory and Dr. Frisard's office in ILSB, which is also locked.

8.4 WHO WILL HAVE ACCESS TO STUDY DATA?


Investigators and graduate students involved in the study.

8.5 DESCRIBE THE PLANS FOR RETAINING OR DESTROYING THE STUDY DATA

The study data will be kept for a period of no longer than 10 years after which they will be shredded or incinerated.

8.6 DOES THIS STUDY REQUEST INFORMATION FROM PARTICIPANTS REGARDING ILLEGAL BEHAVIOR?

No, go to question 9.1

Yes, answer questions within table 

IF YES

Does the study plan to obtain a Certificate of Confidentiality?

No

Yes (Note: participants must be fully informed of the conditions of the Certificate of Confidentiality within
the consent process and form)

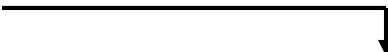
For more information about Certificates of Confidentiality, visit the following link:
<http://www.irb.vt.edu/pages/coc.htm>

Section 9: Compensation

For more information about compensating subjects, visit the following link:
<http://www.irb.vt.edu/pages/compensation.htm>

9.1 WILL SUBJECTS BE COMPENSATED FOR THEIR PARTICIPATION?

No, go to question 10.1

Yes, answer questions within table 

IF YES
What is the amount of compensation? Individuals will receive \$100 for completing the study.
Will compensation be prorated? <input checked="" type="checkbox"/> Yes, please describe: Subjects will be compensated \$50 for completing each muscle biopsy. <input type="checkbox"/> No, explain why and clarify whether subjects will receive full compensation if they withdraw from the study? study?
<i>Unless justified by the researcher, compensation should be prorated based on duration of study participation. Payment must <u>not</u> be contingent upon completion of study procedures. In other words, even if the subject decides to withdraw from the study, he/she should be compensated, at least partially, based on what study procedures he/she has completed.</i>




Section 10: Audio / Video Recording

For more information about audio/video recording participants, visit the following link:
<http://www.irb.vt.edu/pages/recordings.htm>

10.1 WILL YOUR STUDY INVOLVE VIDEO AND/OR AUDIO RECORDING?

No, go to question 11.1

Yes, answer questions within table 

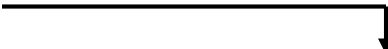
IF YES
This project involves: <input type="checkbox"/> Audio recordings only <input type="checkbox"/> Video recordings only <input type="checkbox"/> Both video and audio recordings
Provide compelling justification for the use of audio/video recording:
How will data within the recordings be retrieved / transcribed?

How and where will recordings (e.g., tapes, digital data, data backups) be stored to ensure security?
Who will have access to the recordings?
Who will transcribe the recordings?
When will the recordings be erased / destroyed?

Section 11: Research Involving Students

11.1 DOES THIS PROJECT INCLUDE STUDENTS AS PARTICIPANTS?

No, go to question 12.1

Yes, answer questions within table 

IF YES
<p>Does this study involve conducting research with students of the researcher?</p> <p><input checked="" type="checkbox"/> No</p> <p><input type="checkbox"/> Yes, describe safeguards the study will implement to protect against coercion or undue influence for participation:</p> <p><i>Note: if it is feasible to use students from a class of students not under the instruction of the researcher, the IRB recommends and may require doing so.</i></p>

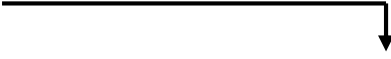
Will the study need to access student records (e.g., SAT, GPA, or GRE scores)?

No

Yes

11.2 DOES THIS PROJECT INCLUDE ELEMENTARY, JUNIOR, OR HIGH SCHOOL STUDENTS?

No, go to question 11.3

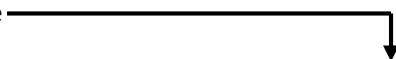
Yes, answer questions within table 

IF YES
<p>Will study procedures be completed during school hours?</p> <p><input type="checkbox"/> No</p> <p><input type="checkbox"/> Yes</p> <p>If yes,</p> <p style="text-align: center;">Students not included in the study may view other students' involvement with the research during school time as unfair. Address this issue and how the study will reduce this outcome:</p> <p style="text-align: center;">Missing out on regular class time or seeing other students participate may influence a student's decision to participate. Address how the study will reduce this outcome:</p>
<p>Is the school's approval letter(s) attached to this submission?</p> <p><input type="checkbox"/> Yes</p> <p><input type="checkbox"/> No, project involves Montgomery County Public Schools (MCPS)</p> <p><input type="checkbox"/> No, explain why:</p>

You will need to obtain school approval (if involving MCPS, click here: <http://www.irb.vt.edu/pages/mcps.htm>). Approval is typically granted by the superintendent, principal, and classroom teacher (in that order). Approval by an individual teacher is insufficient. School approval, in the form of a letter or a memorandum should accompany the approval request to the IRB.

11.3 DOES THIS PROJECT INCLUDE COLLEGE STUDENTS?

No, go to question 12.1

Yes, answer questions within table 

IF YES
<p>Some college students might be minors. Indicate whether these minors will be included in the research or actively excluded:</p> <p><input type="checkbox"/> Included</p> <p><input checked="" type="checkbox"/> Actively excluded, describe how the study will ensure that minors will not be included: Driver's License check</p>
<p>Will extra credit be offered to subjects?</p> <p><input checked="" type="checkbox"/> No</p> <p><input type="checkbox"/> Yes</p> <p>If yes,</p> <p>What will be offered to subjects as an equal alternative to receiving extra credit without participating in this study?</p> <p>Include a description of the extra credit (e.g., amount) to be provided within question 9.1 ("IF YES" table)</p>

Section 12: Research Involving Minors

12.1 DOES THIS PROJECT INVOLVE MINORS (UNDER THE AGE OF 18 IN VIRGINIA)?

Note: age constituting a minor may differ in other States.

No, go to question 13.1

Yes, answer questions within table



IF YES	
Does the project reasonably pose a risk of reports of current threats of abuse and/or suicide?	
<input type="checkbox"/> No	
<input type="checkbox"/> Yes, thoroughly explain how the study will react to such reports:	
<i>Note: subjects and parents must be fully informed of the fact that researchers must report threats of suicide or suspected/reported abuse to the appropriate authorities within the Confidentiality section of the Consent, Assent, and/or Permission documents.</i>	
Are you requesting a waiver of parental permission (i.e., parent uninformed of child's involvement)?	
<input type="checkbox"/> No, both parents/guardians will provide their permission, if possible.	
<input type="checkbox"/> No, only one parent/guardian will provide permission.	
<input type="checkbox"/> Yes, describe below how your research meets all of the following criteria (A-D):	
Criteria A - The research involves no more than minimal risk to the subjects:	
Criteria B - The waiver will not adversely affect the rights and welfare of the subjects:	
Criteria C - The research could not practicably be carried out without the waiver:	


<p>Criteria D - (Optional) Parents will be provided with additional pertinent information after participation:</p>
<p>Is it possible that minor research participants will reach the legal age of consent (18 in Virginia) while enrolled in this study?</p> <p><input type="checkbox"/> No</p> <p><input type="checkbox"/> Yes, will the investigators seek and obtain the legally effective informed consent (in place of the minors' previously provided assent and parents' permission) for the now-adult subjects for any ongoing interactions with the subjects, or analysis of subjects' data? If yes, explain how:</p> <p><i>For more information about minors reaching legal age during enrollment, visit the following link: http://www.irb.vt.edu/pages/assent.htm</i></p>
<p><i>The procedure for obtaining assent from minors and permission from the minor's guardian(s) must be described in Section 4 (Consent Process) of this form.</i></p>

Section 13: Research Involving Deception

For more information about involving deception in research and for assistance with developing your debriefing form, visit our website at <http://www.irb.vt.edu/pages/deception.htm>

13.1 DOES THIS PROJECT INVOLVE DECEPTION?

No, go to question 14.1

Yes, answer questions within table 

IF YES

Describe the deception:
Why is the use of deception necessary for this project?
Describe the debriefing process:
<p>Provide an explanation of how the study meets <u>all</u> the following criteria (A-D) for an alteration of consent:</p> <p>Criteria A - The research involves no more than minimal risk to the subjects:</p> <p>Criteria B - The alteration will not adversely affect the rights and welfare of the subjects:</p> <p>Criteria C - The research could not practicably be carried out without the alteration:</p> <p>Criteria D - (Optional) Subjects will be provided with additional pertinent information after participation (i.e., debriefing for studies involving deception):</p> <p><i>By nature, studies involving deception cannot provide subjects with a complete description of the study during the consent process; therefore, the IRB must allow (by granting an alteration of consent) a consent process which does not include, or which alters, some or all of the elements of informed consent.</i></p> <p><i>The IRB requests that the researcher use the title "Information Sheet" instead of "Consent Form" on the document used to obtain subjects' signatures to participate in the research. This will adequately reflect the fact that the subject cannot fully consent to the research without the researcher fully disclosing the true intent of the research.</i></p>

Section 14: Research Involving Existing Data

14.1 WILL THIS PROJECT INVOLVE THE COLLECTION OR STUDY/ANALYSIS OF EXISTING DATA DOCUMENTS, RECORDS, PATHOLOGICAL SPECIMENS, OR DIAGNOSTIC SPECIMENS?

Please note: it is not considered existing data if a researcher transfers to Virginia Tech from another institution and will be conducting data analysis of an on-going study.

No, you are finished with the application

Yes, answer questions within table



IF YES
From where does the existing data originate?
Provide a detailed description of the existing data that will be collected or studied/analyzed:
Is the source of the data public? <input type="checkbox"/> No, continue with the next question <input type="checkbox"/> Yes, you are finished with this application
Will any individual associated with this project (internal or external) have access to or be provided with existing data containing information which would enable the identification of subjects: <ul style="list-style-type: none">▪ Directly (e.g., by name, phone number, address, email address, social security number, student ID number), or▪ Indirectly through study codes even if the researcher or research team does not have access to the master list linking study codes to identifiable information such as name, student ID number, etc or▪ Indirectly through the use of information that could reasonably be used in combination to identify an individual (e.g., demographics) <input type="checkbox"/> No, collected/analyzed data will be completely de-identified <input type="checkbox"/> Yes, If yes,

Research will not qualify for exempt review; therefore, if feasible, written consent must be obtained from individuals whose data will be collected / analyzed, unless this requirement is waived by the IRB.

Will written/signed or verbal consent be obtained from participants prior to the analysis of collected data? -select one-

This research protocol represents a contract between all research personnel associated with the project, the University, and federal government; therefore, must be followed accordingly and kept current.

Proposed modifications must be approved by the IRB prior to implementation except where necessary to eliminate apparent immediate hazards to the human subjects.

Do not begin human subjects activities until you receive an IRB approval letter via email.

It is the Principal Investigator's responsibility to ensure all members of the research team who interact with research subjects, or collect or handle human subjects data have completed human subjects protection training prior to interacting with subjects, or handling or collecting the data.

-----END-----

Appendix B: Informed Consent for Subjects

Informed Consent for Participants of Investigative Projects Department of Human

Nutrition, Foods and Exercise

Virginia Tech

TITLE: Mitochondrial Autophagy and Endurance

Training INVESTIGATORS: Madlyn I. Frisard, Ph.D.
Kevin P. Davy, Ph.D.
Mathew W. Hulver, Ph.D. Mike Tarpey, M.S.

MEDICAL DIRECTOR: Jose Rivero, M.D.

PURPOSE:

Diseases like obesity and diabetes are associated with a reduction in the processes involved in breaking down damaged or old cell components, which is known as autophagy. Endurance training (running/ cycling) is known for its beneficial effects including reducing body weight and protection from diseases like diabetes, however how it impacts autophagy is currently not known. The purpose of this study is to determine whether autophagy in skeletal muscle is different in endurance trained individuals when compared to sedentary individuals following an overnight fast and in response to a meal.

METHODS:

You are being asked to be involved in a study that involves eating a high fat meal (for example, two sausage and egg biscuits) on one occasion. The study will require a total of 2 visits to the lab.

During session one we will determine your suitability and safety for the study. The full details are outlined further below, but a small amount of blood will be taken to measure several determinants of health such as cholesterol. To determine your current level of fitness you will be asked to complete a VO₂max test designed to measure your maximal oxygen consumption (details below under session one).

Muscle biopsies will be taken at two time points during your second visit to the lab, before the high fat meal and approximately 4 hours after the high fat meal.

If you agree to be involved in this study you will first have to fill out a health history questionnaire. The additional tests are described below under session one. Your results may be discussed with the study medical director to determine if you can be a subject. You may be able to be a subject if you are between 18 and 45 years of age and your body mass index (a measure of obesity) is less than 30 kg/m². If you smoke or have high blood pressure, heart disease or diabetes you cannot be in this study. You will also not be able to participate if your cholesterol is too high or have other health

problems that would make it unsafe for you to be in the study, or if you have lost or gained more than 5 pounds in the last 6 months, or if you use any medication or nutritional

supplements that might influence the study variables. Finally, you will not be able to participate if you have an allergy to lidocaine or bipivacaine, or have food allergies (for example, gluten allergy).

You will complete each study session once. The number and order of the sessions may be changed depending on your time and schedule.

Session 1

Approximate time required: 3 hours

- **Overnight Fast:** You will be asked to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process.
- **Medical History:** You will be asked to complete a medical history questionnaire. This questionnaire is used to screen for health problems or reasons you should not participate in this study. Your height and weight will also be measured at this time. Your body weight will be measured on a standard digital scale. Your height will be measured with a standard stadiometer (ruler on the wall). Your waist, hip, and neck circumference will be measured using a measuring tape.
- **Blood Pressure:** You will be asked to sit quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.
- **Physical Activity Questionnaire:** You will be asked a series of questions to estimate your usual physical activity level, which will require about 15 minutes to complete.
- **Catheter and Blood Draw:** A small needle will be inserted in your arm to draw blood (approximately 3 tablespoons). We will measure glucose, cholesterol, blood cells, liver and kidney enzymes and other factors to determine your eligibility.
- **Body Composition:** This test is to measure your body fat. You will lie on a hospital-type bed and a small amount of x-ray will be passed through your body to determine the amount of bone, muscle and fat in your body. This unit is called a DEXA scan. This test takes approximately 5 minutes and there is no pain associated with the procedure. Your weight and height will also be measured at this time.
- **Maximal Oxygen Consumption:** Maximal oxygen consumption will be measured while exercising on a treadmill. Before the test begins you will be asked to warm-up for 10-15 minutes at a comfortable speed on a treadmill. You will then be asked to run or walk at a fast speed and then the angle of the treadmill will be increased every one or two minutes until you cannot exercise any longer. You will be fitted with a mouthpiece and nose clip so that we can collect and measure the amount of oxygen and carbon dioxide your breath in and exhale out. The test will take approximately 8-12 minutes. Following the test we will have you cool-down for 10-15 minutes at a comfortable walking speed.

Take-Home Tests

- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day) as part of baseline screening.

Session 2

Approximate time required: 5 hours.

- **Overnight Fast:** You will need to avoid eating or drinking for 12 hours and having caffeine- containing foods or drinks for 24 hours before to this visit. This is to make sure that your eating does not influence the test results.
- **Infection/Inflammation Questionnaire:** You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.

- **Meal Challenge:** You will be asked to eat a test meal consisting of two breakfast sandwiches (e.g., egg and sausage). Blood will be collected before and 1, 2, 3, and 4 hours after the meal and a muscle biopsy will be performed before and approximately 4 hours after the meal.

Muscle Biopsy: Two muscle biopsies will be completed, one before and one after the meal challenge (see below). You should not take aspirin, ibuprofen or other non-steroidal, anti-inflammatory medication (such as Advil, Motrin, Celebrex, or Vioxx, or other medication or anything that may affect bleeding or bruising, for 72 hours prior and after this procedure. This procedure is used to sample a small amount of muscle (about 450 mg or about the size of 2 or 3 erasers on a pencil) from below the skin on your thigh. The actual biopsy site will be on the top of either the right or left leg between your knee and hip.

This procedure will be performed by a co-investigator (Kevin P. Davy, Ph.D. or Mathew Hulver, Ph.D.) who has been trained to perform the biopsy. Neither a physician nor nurse may be present during the procedure. You will be lying down and your skin will be cleansed with iodine-type solution (Providine or Betadine). If you are allergic to iodine, we will use chlorhexadine, which does not contain iodine. A sterile drape will be placed over the area and your skin and muscle tissue will be numbed by injecting numbing medication (lidocaine/bipivacaine) into the area with a small needle. If you are allergic to lidocaine or bipivacaine, you cannot participate in this study. Then, a small incision (about 1/4 of an inch) will be made in the skin and a needle (a little thinner than a pencil) will be inserted to remove a small amount of muscle (about 450 mg or 1 teaspoon). Some suction may be applied to the other end of the needle to help remove the muscle.

After the biopsy is completed, pressure will be applied and the skin will be closed with sterile tape. To ensure cleanliness, the skin will be cleaned with saline and will be covered with gauze and a clear adhesive dressing. The site will then be wrapped with an ACE bandage. You will be asked to keep the ACE bandage on for at least 10-15 minutes. You may take Tylenol for any discomfort you may experience following the biopsy. We will use the biopsy samples to measure factors which contribute to inflammation. The biopsy will take place at either the Human Integrative Physiology Laboratory (228 War Memorial Hall) or Dr. Jose Rivero's medical office in Christiansburg. You will be asked to return to the physiology laboratory within 5 days after the biopsy to have the site checked to ensure proper healing.

You will be provided with instructions on how to care for the biopsy sites as well as what to look for if a problem were to occur.

- **Catheter and Blood Draw:** A catheter (small plastic tube) will be inserted in your arm to draw blood (approximately 3 tablespoons). We will measure various hormones that influence your metabolism (how your body burns calories and produces body heat). Blood will be collected before the meal challenge and at 1, 2, 3, and 4 hours after the meal. The catheter will remain in your arm throughout the entire test.

SUMMARY OF SUBJECT RESPONSIBILITIES

- Provide an accurate history of any health problems or medications you use before the study begins.
- Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.
- Be on time and attend all of the scheduled experiments.
- Follow all participant instructions for each session.

- Follow diet and physical activity instructions provided by the investigators.

RISKS OF PARTICIPATION

- **Catheter and Blood Draw:** Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10 or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood loss is 1 in 1000. There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the catheter is removed. If you feel faint during or after a blood draw, you should notify the study doctor or study staff immediately and lie down right away to avoid falling down. Having staff experienced in catheter placement and blood draws will minimize these risks.
- **HIV/ Hepatitis B/ Hepatitis C:** In the event a researcher or other staff person is improperly exposed to your blood, your blood will be tested for the presence of HIV, the Hepatitis B Virus, and the Hepatitis C Virus. There will not be any cost to you for this test. The research team will follow proper procedures for testing and reporting as outlined by Virginia State Law, which includes sending the sample to a certified laboratory. Please note that, should your blood require testing, you will be informed of your test results and provided with the opportunity to receive appropriate and timely counseling. In addition, your results will be sent to the local health department.
- **Muscle Biopsies:** If you are allergic to lidocaine, you will not be allowed to participate in this study. There may be slight discomfort and burning when the local anesthetic is injected prior to the biopsy, but you are not expected to experience discomfort during the biopsy procedure. Bruising in the area of the muscle biopsy for 1-2 weeks will likely occur, but local pressure and ice are applied to the site immediately after the procedure to limit this potential effect and its accompanying tenderness. There is a slight risk of infection at the biopsy site. There is a small risk that you will become lightheaded, dizzy, or anxious before or during the procedures. All of these reactions are temporary and resolve within a short time after completing or stopping the procedure. These risks are minimized by having a trained individual perform the procedure. You will be asked to return to the physiology laboratory within 5 days after the biopsy to have the site checked to ensure proper healing.

You will likely receive a scar from each of the biopsies performed but these are expected to be very small. These scars usually turn a purple color in the weeks to months following the biopsy and then fade considerably over time. The study staff will show you several pictures of examples of the scarring (greater than 1 year old) that can occur following similar biopsy procedures. It is important that you understand that these are just examples of the scarring that can occur. The actual scar you receive may be smaller or larger or differ in coloring.

Individuals with darker skin (e.g., African Americans, Hispanics and Asians) tend to scar more than those with lighter skin. You should consider this before you agree to participate.

There is a small risk that you will become lightheaded, dizzy, or anxious before, during or after the procedure. There is also a small risk of fainting. If you feel dizzy, lightheaded or feel like you might faint before, you should sit down or lie down immediately to avoid falling. These reactions are usually temporary and resolve within a short time after sitting or lying down. If these feeling do not go away soon after sitting or lying down, you should call 911 or have someone take you to the nearest emergency room. All of these reactions are temporary and resolve within a short time after completing or stopping the procedure. We did have one individual faint and hit their head after leaving the laboratory. This required a trip to the emergency room and stitches. However, this occurred only once

in the over 350 biopsies

performed in our research studies.

- **DEXA Scan:** The amount of radiation that you will receive in the DEXA exam is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount you will receive is equal to 1/20 of a chest x-ray. The more radiation you receive over the course of your lifetime, the more likely your risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks; however the exact increase in such risk is not known.
- **Maximal Oxygen Consumption:** There is a small risk of injury (e.g., sprained ankle), complications requiring you to go to the hospital, heart attack, or even death. In studies involving people with heart disease, the risk of hospitalization was 1 in 500 tests (<0.20%). The risk of heart attack was 1 in 2,500 tests (0.04%) and death, 1 in 10,000 tests (0.01%). The risks are likely to be lower in young, healthy subjects. Only experienced staff members will conduct these tests and you will be monitored throughout the test for signs of problems. You will be tired after this test and may have sore muscles for a few days.
- It is not possible to identify all potential risks in an experiential study. However, the study doctors and study staff will take all possible safeguards to minimize any known and potential risks to your well-being. We believe the overall risks of participation are minimal. All of the procedures are well established and used routinely in the study investigators laboratory.
- Side effects are possible in any research study despite high standards of care, and could occur through no fault of your own or the study doctors or study staff.

BENEFITS OF PARTICIPATION

Your participation will provide you with:

- Information on your body composition.
- Information on your blood pressure, cholesterol and glucose tolerance.
- Information on your maximal oxygen consumption.

COMPENSATION

You will be compensated \$50 for completing each muscle biopsy. Muscle biopsies will be performed before and after the test meal during session 2. Total compensation for your participation in the study is \$100.

CONFIDENTIALITY

The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by subject numbers, without anything to identify you by name. In the event that any of your tests indicate that you may have a heart problem, Dr. Rivero or investigators may want to share this information with your doctor but he will request your approval first.

FREEDOM TO WITHDRAW

You are free to withdraw from the study at any time for any reason. Simply inform the experimenters of your intention to cease participation. In addition, circumstances could arise which would lead to your exclusion from the study. For example, lack of compliance to instructions, failure to attend testing sessions, and illness could be reasons for the researchers to stop your participation in the study. Other reasons include an inability by the researchers to obtain muscle, body fat or other measurements that are necessary for the study.

INJURY DURING PARTICIPATION IN THIS STUDY

Neither the researchers nor the University have money set aside to pay for medical treatment that would be necessary if injured as a result of your participation in this study. Any expenses that you incur including emergencies and long-term expenses would be your own responsibility. You should consider this limitation before you consider participating in this study.

APPROVAL OF RESEARCH

This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech. You will receive a copy of this form to take with you.

SUBJECT PERMISSION

I have read the informed consent and fully understand the procedures and conditions of the project. I have had all my questions answered, and I hereby give my voluntary consent to be a participant in this research study. I agree to abide by the rules of the project. I understand that I may withdraw from the study at any time.

If you have questions, you may contact:

- Principal Investigator: Madlyn Frisard, Assistant Professor, Department of Human Nutrition, Foods, and Exercise. (540) 231-9994; After hours: (540) 818-9907

- Co-Investigator: Kevin Davy, Professor, Department of Human Nutrition, Foods, and Exercise. (540) 231-3487; After hours: (540) 230-0486

- Chairman, Institutional Review Board for Research Involving Human Subjects: David Moore, Associate Vice President for Research (540) 231-4991

Name of Subject (please print)_____

Signature of Subject_____ Date_____

Appendix C: Health History Questionnaire

Virginia Tech
Department of Human Nutrition, Foods, and Exercise

HEALTH HISTORY QUESTIONNAIRE

STUDY _____ DATE _____

SUBJECT ID # _____

PLEASE PRINT

1. Address: _____
City: _____ State: _____ Zip Code _____
Home Phone: _____ Work Phone: _____
E-mail address: _____
Emergency Contact: _____ Phone: _____
Relation to you: _____

2. Employer: _____ Occupation: _____

3. Age: _____ Sex: _____

Race and/or Ethnic Origin

- American Indian or Alaskan Native Asian or Pacific Islander Black, not of Hispanic Origin
 Hispanic White, not of Hispanic Origin
 Other

4. GENERAL MEDICAL HISTORY

Do you have any current medical conditions? YES NO If Yes, please explain:

Are you allergic to any medications? YES NO If Yes, please explain:

Have you had any major illnesses in the past? YES NO If Yes, please explain:

Have you ever been hospitalized or had surgery? YES NO If Yes, please explain:
(include date and type of surgery, if possible)

Are you currently taking any medications or supplements, including aspirin, hormone replacement therapy, or other over-the-counter products?

YES NO If Yes, please explain:

Medication/Supplement **Reason** **Times taken per Day** **Taken for how long?**

Have you ever had an EKG? YES NO If Yes, please explain:

Have you been diagnosed with diabetes? YES NO If Yes, please explain:

Age at diagnosis_____

FAMILY HISTORY

	Age (if alive)	Age of Death	Cause of Death
Father	_____	_____	_____
Mother	_____	_____	_____
Brothers/Sisters	_____	_____	_____
	_____	_____	_____
	_____	_____	_____
	_____	_____	_____

Do you have a family history of any of the following: (Blood relatives only, please give age at diagnosis if possible)

	YES	NO	Relation	Age at Diagnosis
a. High blood pressure	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____
b. Heart Attack	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____
c. Coronary bypass surgery	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____
d. Stroke	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____
e. Diabetes	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____
f. Obesity	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____

6. **TOBACCO/ALCOHOL HISTORY** (check one)

- None
- Quit (when) _____
- Cigarette
- Cigar
- Pipe
- Chew Tobacco
- Snuff

CURRENT TOBACCO USE

- (if applicable)
- # per day**
- Cigarette _____
 - Cigar _____
 - Pipe _____
 - Chew Tobacco _____
 - Snuff _____

Total years of tobacco use _____

Do you consume alcohol? Drinks per day ____ Drinks per week ____

7. CARDIORESPIRATORY/METABOLIC HISTORY

	YES	NO
Are you presently diagnosed with heart disease?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have any history of heart disease?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have a heart murmur?	<input type="checkbox"/>	<input type="checkbox"/>
Occasional chest pain or pressure?	<input type="checkbox"/>	<input type="checkbox"/>
Chest pain or pressure on exertion?	<input type="checkbox"/>	<input type="checkbox"/>
Episodes of fainting?	<input type="checkbox"/>	<input type="checkbox"/>
Daily coughing?	<input type="checkbox"/>	<input type="checkbox"/>
High blood pressure?	<input type="checkbox"/>	<input type="checkbox"/>
Shortness of breath?		
At rest?	<input type="checkbox"/>	<input type="checkbox"/>
lying down?	<input type="checkbox"/>	<input type="checkbox"/>
After 2 flights of stairs?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have asthma?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have a history of bleeding disorders?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have a history of problems with blood clotting?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have high cholesterol? Or, low good (HDL) cholesterol?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have thyroid problems?	<input type="checkbox"/>	<input type="checkbox"/>

If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.

8. **MUSCULOSKELETAL HISTORY**

	YES	NO
Any current muscle injury or illness?	<input type="checkbox"/>	<input type="checkbox"/>
Any muscle injuries in the past?	<input type="checkbox"/>	<input type="checkbox"/>
Do you experience muscle pain at rest?	<input type="checkbox"/>	<input type="checkbox"/>
Do you experience muscle pain on exertion?	<input type="checkbox"/>	<input type="checkbox"/>
Any current bone or joint (including spinal) injuries?	<input type="checkbox"/>	<input type="checkbox"/>
Any previous bone or joint (including spinal) injuries?	<input type="checkbox"/>	<input type="checkbox"/>
Do you ever experience painful joints?	<input type="checkbox"/>	<input type="checkbox"/>
Do you ever experience swollen joints?	<input type="checkbox"/>	<input type="checkbox"/>
Do you ever experience edema (fluid build up)?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have pain in your legs when you walk?	<input type="checkbox"/>	<input type="checkbox"/>

If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.

8. **NUTRITIONAL HABITS**

Do you have any food allergies? YES NO If Yes, please explain:

Have you ever dieted? YES NO

If YES, have you dieted within the past 12 months or are you currently on a diet?

YES NO

If YES, please describe the diet:

a). Name (if applicable): _____

b). Prescribed by a Physician/nutritionist? YES NO

c). Have you lost weight? YES NO

d). Duration of diet _____

8. **NUTRITIONAL HABITS (CON'T)**

What was your weight 24 months ago? _____ 12 months ago? _____ 6 months ago? _____

Have you dieted other than in the past 12 months? YES NO

If YES, please answer the following:

- a). How many times have you dieted?
- b). How old were you?
- c). Weight loss (amount)?

You may be asked to complete a more detailed diet survey if you are volunteering for a research study.

9. PHYSICAL ACTIVITY SURVEY

Compared to a year ago, how much regular physical activity do you get? (Check one)

- Much less
- Somewhat less
- About the same
- Somewhat more
- Much more

Have you been exercising regularly for the past three months? YES NO

If YES, what type of exercise do you regularly participate in? (check those that apply)

	Days per week	Minutes per session	Intensity (1=easy, 10=very hard)
Walking <input type="checkbox"/>	_____	_____	_____
Running <input type="checkbox"/>	_____	_____	_____
Cycling <input type="checkbox"/>	_____	_____	_____
Swimming <input type="checkbox"/>	_____	_____	_____
Aerobics <input type="checkbox"/>	_____	_____	_____
Weight Training <input type="checkbox"/>	_____	_____	_____
Martial Arts <input type="checkbox"/>	_____	_____	_____
Other (describe) <input type="checkbox"/>	_____	_____	_____

10. **SLEEP HISTORY**

Please answer yes/no or circle appropriate answer.

Do you snore?

YES

NO

Don't Know

Snoring loudness

Loud as breathing

Loud as talking

Louder than talking

Very loud. Can be heard in nearby rooms.

Snoring frequency

Almost every day

3-4 times per week

1-2 times per week

1-2 times per month

Never or almost never

Does your snoring bother other people?

Has anyone told you that you quit breathing during your sleep?

How often have your breathing pauses been noticed?

Almost every day

3-4 times per week

1-2 times per week

1-2 times per month

Never or almost never

Are you tired after sleeping?

Almost every day

3-4 times per week

1-2 times per week

1-2 times per month

Never or almost never

Are you tired during waketime?

Almost every day

3-4 times per week

1-2 times per week

1-2 times per month

Never or almost never

Have you ever fallen asleep while driving?

Almost every day

3-4 times per week

1-2 times per week

1-2 times per month

Never or almost never

Sleepiness Assessment

- 0 (zero) = would never doze off
- 1 (one) = slight chance of dozing
- 2 (two) = moderate chance of dozing
- 3 (three) = high chance of dozing

Situation

Chance of Dozing

Sitting and reading	___
Watching TV	___
Sitting, inactive in a public place (e.g., a theatre or meeting)	___
As a passenger in a car for an hour without a break	___
Lying down to rest in the afternoon when circumstances permit	___
Sitting quietly after lunch without alcohol	___
Sitting and talking to someone	___
In a car, while stopped for a few minutes in traffic	___

11. EDUCATION

Please check the highest degree obtained:

- Grade School
- Junior High
- High School
- College Degree
- Master's Degree
- Doctorate

12. FAMILY PHYSICIAN

Name: _____

Address: _____

Phone: _____

Should it be necessary, may we send a copy of your results to your physician? YES NO

Signature: _____

Date: _____

Witness: _____

Date: _____

Print Name Signature

Reviewer: _____

Date: _____

Print Name Signature

Appendix D: Physical activity Questionnaire

Godin Leisure-Time Exercise Questionnaire

1. During a typical 7-Day period (a week), how many times on the average do you do the following kinds of exercise for more than 15 minutes during your free time (write on each line the appropriate number).

- | | Times Per Week |
|---|----------------|
| a) STRENUOUS EXERCISE
(HEART BEATS RAPIDLY) | _____ |
| (e.g., running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling) | |
| b) MODERATE EXERCISE
(NOT EXHAUSTING) | _____ |
| (e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing) | |
| c) MILD EXERCISE
(MINIMAL EFFORT) | _____ |
| (e.g., yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking) | |

2. During a typical 7-Day period (a week), in your leisure time, how often do you engage in any regular activity long enough to work up a sweat (heart beats rapidly)?

1. Often _____ 2. Sometimes _____ 3. Rarely/Never _____

Appendix E: Infection and Inflammation Questionnaire

INFECTION/INFLAMMATION QUESTIONNAIRE

Evaluator Script: I would like you to think if you had a cold, the flu, a dental infection or other infection during the past month. I am going to ask you about some symptoms that may have accompanied those types of conditions.

1) Did you have a cold, the flu, a dental infection or other infection in the past month?

Yes No Refused Don't Know

If yes, Within 1 week 2 weeks prior 3 weeks prior 4 weeks prior

In the prior month did you experience any of the following symptoms? [Note to examiner: If symptom was present, the timing of symptom onset and resolution (# days) prior to interview is recorded. If symptom is still present on the day of interview, place 0 in "Resolved ___ days ago".]

2) Did you feel feverish or have a fever? Yes No

If Yes, Symptom Started ___ days ago. Resolved ___ days ago.

Did you take your temperature? Yes No

3) Chills? Yes No

If Yes, Started ___ days ago. Resolved ___ days ago.

4) Sore throat ? Yes No

If Yes, Started ___ days ago. Resolved ___ days ago.

5) Coughing? Yes No

If Yes, Started ___ days ago. Resolved ___ days ago.

6) Sputum? Yes No

If Yes, Started ___ days ago. Resolved ___ days ago.

7) Sneezing? Yes No

If Yes, Started ___ days ago. Resolved ___ days ago.

8) Runny nose or nasal congestion? () Yes () No

If Yes, Started ___ days ago. Resolved ___ days ago.

If Yes to (5), (6), (7), or (8). Do you have seasonal allergies? () Yes () No

Do you have a chronic lung or sinus condition? () Yes () No

If Yes, are these symptoms typical for your chronic lung or sinus condition?

() Yes () No

9) Ear pain or discharge? () Yes () No

If Yes, Started ___ days ago. Resolved ___ days ago.

10) Run down feeling or achy muscles you feel may have been due to a cold or flu?

() Yes () No

If Yes, Started ___ days ago. Resolved ___ days ago.

11) Tooth/Gum pain? () Yes () No

If Yes, Started ___ days ago. Resolved ___ days ago.

If Yes, did you seek dental care? () Yes () No

If Yes, did a Dentist find a cavity or other dental infection? () Yes () No

12) Mouth/gum (Y N), Skin (Y N), or Joint (Y N) redness or swelling?

If Yes, Started ___ days ago. Resolved ___ days ago.

13) Skin infection? () Yes () No

If Yes, Started ___ days ago. Resolved ___ days ago.

14) Nausea/Vomiting? () Yes () No

If Yes, Started ___ days ago. Resolved ___ days ago.

15) Diarrhea? () Yes () No

If Yes, Started ___ days ago. Resolved ___ days ago.

16) Pain upon urination or urgency? () Yes () No

If Yes, Started ___ days ago. Resolved ___ days ago.

17) Cloudy discolored urine? ()Yes ()No
Urinalysis showing evidence of infection? ()Yes ()No
If Yes, Started ___ days ago. Resolved ___ days ago.

18) Did you seek medical care for any sort of cold, flu, or infection in the prior month?
()Yes ()No
If yes, diagnosis given_____

19) Did you take any over the counter or prescription medications for a cold, flu, or any
infection in the prior month?
()Yes ()No
If yes, names of medication_____
