Longer Life Foundation: Final Report
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Project Title: Is Aging and its Associated Co-morbidites Due to Diminished Autophagy? (2012)

Abstract:
Autophagy, or "self-eating", is a mechanism by which cells and tissues recycle damaged protein and organelles. Recent studies suggest that autophagy is protective against aging and aging-related diseases. In addition, loss of autophagy leads to many diseases associated with aging, including neurodegeneration and muscle atrophy in model organisms. However, little is known about the rates of autophagic degradation in human tissues. This is because no reliable assay has been developed to selectively measure autophagic degradation *in vivo* in human patients. This research sought to develop and validate a novel *in vivo* autophagic degradation assay using stable isotope labeling and tandem mass spectrometry of the autophagic substrate p62. Upon completion of this study, we hope to be able to move directly into human patients and evaluate rates of autophagic degradation in select tissues, beginning with skeletal muscle.

Lay Summary:
Cells and tissues have developed many adaptive response that mediate longevity and healthspan. One of these is autophagy or "self-eating," a mechanism whereby cells and tissues recycle damaged protein and organelles. Autophagy may protect against aging and aging-related diseases. In addition, loss of autophagy leads to many diseases associated with aging, including neurodegeneration and muscle atrophy in model organisms. However, little is known about autophagy in human tissues because no reliable assay exists to measure autophagy reliably in human patients. This research sought to develop and validate a novel *in vivo* autophagic degradation assay using stable isotope labeling and tandem mass spectrometry. We hope that this method will be able to ultimately be used in human patients to measure rates of autophagy.

Introduction:
The biochemical pathways associated with longevity and life span (e.g. caloric restriction, mTOR, Sirt1 and insulin growth factor signaling) converge on the regulation of autophagy in invertebrate and vertebrate models [1]. Autophagy is a tightly-controlled proteolytic system that degrades damaged proteins and organelles, resulting in the liberation of free amino acids and remodeling of cellular contents. During times of nutrient (i.e. caloric restriction) or growth factor deprivation, autophagic pathways are upregulated in cells. Aged tissue has a diminished autophagic response, suggesting that autophagy serves a protective role in tissue homeostasis. Loss of autophagy in specific tissues leads to age-associated disease phenotypes such as sarcopenia, heart failure, neurodegeneration and malignancy in mouse models [2-6]. In aging humans, diminished autophagy in skeletal muscle has been associated with muscle wasting or sarcopenia [7]. Other studies in animal models and humans implicate diminished autophagic function in other age-associated co-morbid conditions including osteoarthritis, hepatosteatosis, obesity, diabetes and metabolic syndrome [8-10].

It is becoming clear that augmenting or activating autophagy is lifespan-promoting and protective. For example, treatment of inbred mice with the drug rapamycin (which activates autophagy) extends their life spans [11]. In addition, mouse models of osteoarthritis and some neurodegenerative disorders improve when autophagy is activated with drug treatment [12-14]. Recent evidence suggests that the extended health benefits of physical activity are mediated through an activation of autophagy in skeletal muscle during exercise that modulates glucose utilization and insulin sensitivity [15]. Exercised obese-diabetic mice deficient in skeletal muscle autophagy remain obese and diabetic compared with exercised obese-diabetic mice with normal muscle autophagy [15].

Whether autophagy mediates longevity and organismal health span in humans is not known. It is also not clear whether aged individuals have a decrease in autophagic capacity compared with younger individuals or whether humans with higher rates of autophagy in skeletal muscle are more protected from metabolic complications. Finally, whether therapies aimed at enhancing autophagy will protect against senescence and its co-morbidities is not established.

Currently, the greatest limitation to understanding autophagic processes in human health and disease is the lack of reliable quantitative assays to measure autophagic degradation in vivo. The main proteolytic pathways in cells and tissue are the ubiquitin-proteasome system (UPS) and autophagy. Unfortunately, measuring total protein degradation cannot distinguish between these two pathways. Therefore one must identify a protein that is exclusively degraded via the autophagic pathway. **Our preliminary studies have confirmed that the autophagic adaptor protein p62 (SQSTM1) is a likely autophagic substrate candidate in skeletal muscle.**

Methods:
We proposed that the in vivo rate of p62 degradation is a direct measure of autophagic pathway activity in human skeletal muscle that can be quantified using stable isotope-labeling tandem mass spectrometry (SILT). The collaboration between Dr. Weihl (a neuromuscular physician interested in protein degradation pathways in skeletal muscle) and Dr. Yarasheski (a pioneer in stable isotope-labeling and mass spectrometry of human skeletal muscle proteins) was uniquely positioned to address this question.

1) Quantify the half-life (t½) of p62 in mouse skeletal muscle via SILT (stable isotope labeling tandem mass spectrometry). Aim 1 will determine the basal rate of autophagy in normal mouse skeletal muscle.

2) Determine the t½ of p62 following the induction and inhibition of autophagy in mouse skeletal muscle following systemic treatment with autophagy-enhancing and -blocking drugs and in genetically-modified mice defective in autophagy. Aim 2 will confirm that p62 is an autophagic substrate and evaluate the *in vivo* dynamics (protein kinetics) of autophagic degradation in skeletal muscle. p62 degradation rate should decrease under conditions of autophagic inhibition and increase when autophagy is activated.

Results:
1) Saturating doses of Leucine inhibit autophagy and diminish p62 gene expression. Our initial studies utilized $^{13}C_6$-leucine as the labeling amino acid. To perform these studies, we administered intraperitoneal $^{13}C_6$-leucine for 5 days. Surprisingly, and somewhat counterintuitive, the administration of a branched-chain amino acid resulted in suppression of the selective protein synthesis of p62. This was of course problematic, since our goal was to identify $^{13}C_6$-leucine incorporation into newly-synthesized p62. Therefore for all subsequent studies, we utilized D_5-phenylalanine.

2) The fractional synthesis rate (FSR) of p62 is ~5%/day or a t½ of 13.5 days in mouse skeletal muscle. Our studies were able to define the t½ of p62 in mouse skeletal muscle. Utilizing the SILT method, we identified three p62 peptides that contained measurable D_5-phenylalanine. The ratio of incorporated D_5-phenylalanine containing p62 peptide/ total p62 peptide was 25% after five days of label. These measures are an invaluable starting point toward the identification of p62 kinetics.

Discussion:
We have now fulfilled our primary study goal of developing a method that can measure rates of autophagy in mouse skeletal muscle. More importantly, our technique can accurately measure the synthetic and catabolic rates of the marker of autophagy, p62, in skeletal muscle.

Thanks to the support obtained through the Longer Life Foundation, we now have demonstrated the feasibility of our stable isotope approach to measure markers of autophagy in muscle and will now be able to move this technique forward into humans. We will be submitting an R21 development grant in the early part of 2014 using the preliminary data generated by this proposal.