

Addendum

Coordinate activation of autophagy and the proteasome pathway by FoxO3 transcription factor

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The rapid loss of muscle mass, which occurs with disuse and systemically with fasting, cancer and many other diseases, results primarily from accelerated breakdown of muscle proteins. In atrophying muscles, the ubiquitin-proteasome pathway catalyzes the accelerated degradation of myofibrillar proteins, but the possible importance of the autophagic/lysosomal pathway in atrophy has received little attention. Our prior studies demonstrate that activation of FoxO transcription factors is essential for muscle atrophy, and that activated FoxO3 by itself causes dramatic atrophy of muscles and cultured myotubes via transcription of a set of atrophy-related genes (“atrogene”) including critical ubiquitin ligases. Using selective inhibitors, we measured isotopically the actual contribution of proteasomes and lysosomes to the FoxO3-induced increase in protein breakdown in myotubes and found that FoxO3 coordinately activates both proteolytic systems, but especially lysosomal proteolysis. Activated FoxO3 stimulates autophagy through a transcription-dependent mechanism and increases the transcription of many autophagy-related genes, which are also induced in mouse muscles atrophying due to denervation or fasting. Thus, in atrophying muscles, decreased IGF1-PI3K-Akt signaling stimulates autophagy, not only through mTOR, but also more slowly by FoxO3-dependent transcription. These findings on muscle provide the first evidence for coordinate regulation of proteasomal and lysosomal systems, although in neuronal and hepatic cells, FoxO3 stimulates the autophagic process selectively.

Muscle atrophy is a debilitating process that leads to rapid loss of strength and endurance. It occurs in specific muscles with inactivity or denervation and occurs systemically in fasting and with many diseases, including cancer, diabetes, sepsis and renal failure.¹ In these various conditions, the rapid loss of muscle mass occurs primarily

through accelerated protein breakdown. Recent studies of this acceleration in proteolysis have provided extensive evidence for activation of the ubiquitin-proteasomal pathway in atrophying muscles.² This pathway catalyzes the degradation of myofibrillar proteins,³ the major protein constituent of adult skeletal muscles. Our lab has previously identified a set of atrophy-specific genes or “atrogene” which are up or downregulated similarly in muscles in diverse types of atrophy.^{4,5} Thus, there exists a common transcriptional program that leads to accelerated protein degradation including induction of multiple components of the ubiquitin-proteasome pathway: several ubiquitin genes, subunits of the proteasome, and the atrophy-specific ubiquitin ligases, *atrogen-1* and *MuRF-1*. However, mRNAs for certain components of the autophagic/lysosomal pathway (including *LC3*, *Gabarrapl1* and *Cathepsin L*) are also found to be upregulated in various types of atrophy. Also, in earlier studies, increased autophagosome formation is observed in muscles after denervation⁶ and during starvation,⁷ and a greater capacity of lysosomal proteolysis is demonstrated during denervation atrophy.⁸ We therefore undertook studies to test whether the autophagic/lysosomal system may also play an important role in muscle atrophy, since it seems likely that these two proteolytic systems might serve complimentary roles in degrading distinct cellular constituents and might be activated coordinately by common signaling mechanisms.

We previously showed that activation of FoxO transcription factors is essential for the fiber atrophy and atrogen-1 induction upon denervation, fasting and glucocorticoid treatment.^{9,10} In growing muscles, when the IGF1/insulin-PI3K-Akt signaling pathway is active, FoxO3 is inactive and sequestered in the cytosol, but in catabolic states, FoxO transcription factors enter the nucleus and become active.¹⁰ To address how FoxO3 causes atrophy, we measured overall rates of proteolysis in C2C12 myotubes by following the degradation of prelabeled long-lived proteins. To measure the contributions of each system, we utilized specific inhibitors of the proteasome's peptidase activity (Velcade, lactacystin) or lysosomal acidification (concanamycin A, NH₄Cl or chloroquine). We find that overexpression of constitutively active FoxO3 (ca-FoxO3), which causes dramatic atrophy by 2 days, enhances protein degradation by more than 50% and increases both proteasomal and lysosomal processes. The stimulation of lysosomal proteolysis is surprisingly large, 3-fold greater than that of proteasomal degradation. This enhanced lysosomal proteolysis is shown to result from activation of autophagy, as evidenced by the lipidation of LC3, the sensitivity of the increased

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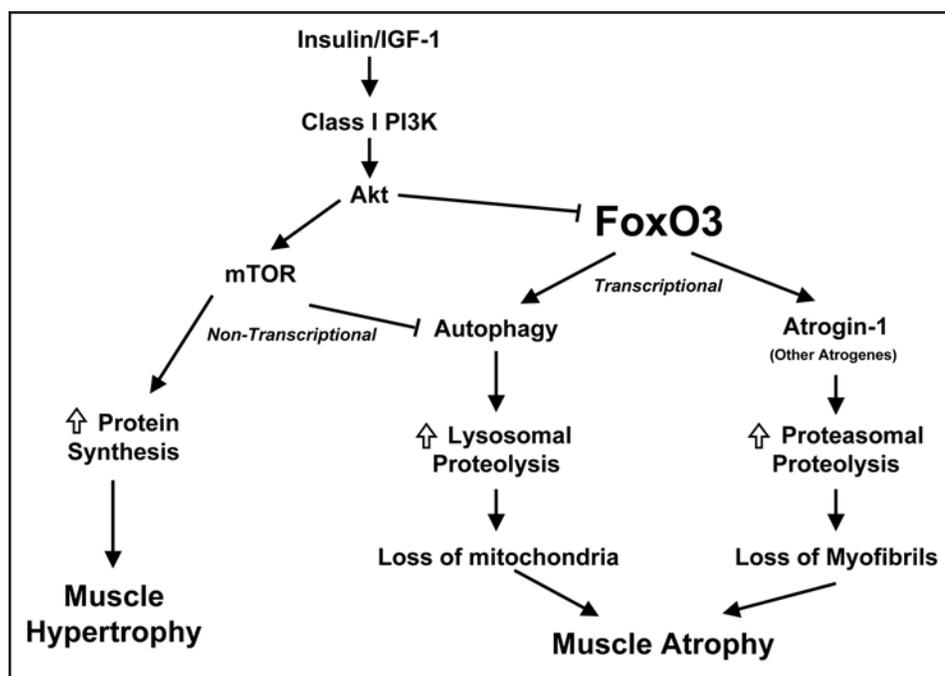


Figure 1. Regulation of muscle protein breakdown by lysosomal and proteasomal pathways during hypertrophy and atrophy.

lysosomal proteolysis to the autophagy inhibitor 3-methyladenine and the increased formation of autophagosomes shown with GFP-LC3 reporter. Also, studies in *Atg5*-deficient cells confirmed the key role of autophagy in this enhanced proteolysis. This stimulation of autophagy requires transcription, in contrast to the smaller increase in lysosomal proteolysis induced with rapamycin. In addition, we find that ca-FoxO3 increases the transcription of many autophagy-related genes, including *LC3b*, *Gabrarap1*, *Atg12l*, *PI3KIII*, *Ulk2*, *Atg4b* and *Beclin1*. In addition, we demonstrate by Chromatin Immunoprecipitation that FoxO3 binds directly to the promoters of *LC3*, *Gabrarap1* and *Atg12l*. By contrast, no evidence of increased chaperone-mediated autophagy is obtained.¹¹

FoxO3 activation seems also to be essential for activation of autophagy in mouse muscles. These same seven autophagy-related genes induced in myotubes by FoxO3 are also induced in mouse muscles atrophying due to denervation or fasting. Also, in isolated muscle fibers, we show that activated FoxO3 increases the number of GFP-LC3-labeled autophagosomes, and that FoxO3 is necessary for starvation-induced enhancement of autophagy.¹¹ At the same time, in a complimentary study, our collaborators, Mammucari et al., reached similar conclusions by different approaches.¹² Together, these studies demonstrate a new mode of regulation of autophagy via FoxO3-dependent transcription and also that the autophagic/lysosomal pathway in muscle is regulated coordinately with the proteasomal pathway (Fig. 1). Both seem to contribute importantly to muscle atrophy and loss of function. Presumably, the ubiquitin proteasome pathway, in catalyzing degradation of myofibrillar components, accounts for the loss of strength and the activation of autophagy accounts for the loss of mitochondria and endurance.

In myotubes, ca-FoxO3 induced seven autophagy-related genes,¹¹ but did not affect some critical *Atg* genes, such as *Atg5*, *Atg7* and *Atg10* (unpublished). It remains unclear whether these

FoxO3-dependent transcriptional changes drive the enhanced lysosomal proteolysis or only support the cells' capacity to maintain high rates of autophagy by replacing components consumed during autophagy (as we found to occur with LC3 and Gabarap1). Perhaps FoxO3 causes expression of an unknown factor, which directly activates autophagy. Mammucari et al present evidence that in adult muscles, the mitochondrial component, Bnip3, which is induced during all types of atrophy studied and by FoxO electroporation, can stimulate autophagy.¹² However, in C2C12 myotubes, FoxO3 does not induce the transcription of either *Bnip3* or *Bnip3L* (unpublished). Thus, in myotubes FoxO3 seems to activate autophagy through additional mechanisms.

Our studies analyze the flux of cell proteins through proteasomes and lysosomes after long-term labeling of cell proteins. By using specific inhibitors at concentrations that maximally block these degradation pathways, we are able to obtain insights that

are not possible by more standard non-quantitative approaches. For example, it is often stated that the lysosomal system is responsible for degradation of long-lived cell proteins, while the ubiquitin-proteasome pathway digests short-lived (regulatory or misfolded) proteins. However, in our control myotube cultures, only about 40% of the degradation of long-lived proteins involves lysosomes while about 50% are degraded by proteasomes. In contrast, 70% of the FoxO3-induced increase in protein breakdown in myotubes requires lysosomal function. In adult muscles, on the other hand, where myofibrils are more abundant, the proteasome is likely to degrade a much greater fraction of cell proteins both in growth and atrophy.³ Thus the relative importance of these two pathways depends on the cell type and varies in different physiological states.

At present, there is appreciable interest in developing pharmacological agents that block muscle wasting (e.g., by inhibition of ubiquitin ligases).¹³ Our data suggest that their inhibition may spare myofibrillar or soluble proteins, but would not prevent loss of proteins degraded by autophagy (e.g., mitochondria). On the other hand, activators of the IGF1-PI3K-Akt pathway should both enhance protein synthesis generally and suppress FoxO activation and thus reduce both proteasomal and lysosomal protein degradation. Overexpression of ca-FoxO3 also accelerates proteolysis in hepatocyte and neuronal cultures by stimulating the lysosomal process, but does not activate the proteasomal pathway, presumably because these cells, unlike muscles, do not contain FoxO-dependent ubiquitin ligases or myofibrils. There has also been appreciable interest in stimulating autophagy as a therapeutic strategy to clear inclusions of abnormal proteins in neurons (e.g., in polyglutamine diseases) using the inhibitor of mTOR, rapamycin. The present studies indicate a distinct mode of activation of autophagy that causes much larger increases in proteolysis than induced by rapamycin treatment. Possibly, this new mode of activation of autophagy

via FoxO3 will offer new approaches to treatment of such diseases resulting from aberrant proteins.

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