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p97's Roles in Muscle Atrophy, Mitochondrial Membrane Protein Turnover, and the Effects of p97 Inhibition

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The valosin-containing protein, p97, is ATP-driven ubiquitin-selective chaperone whose primary function is the extraction and degradation of a wide variety of protein complexes. This microreview will summarize recent studies in the cellular functions of p97 in relation to degradation of muscle proteins, regulation and turnover of outer mitochondrial membrane, and the effects of p97 inhibition.

Introduction

p97, also known as valosin-containing protein (VCP) in higher eukaryotes or Cdc48p in yeast, is a phylogenetically convserved member of the type II ATPases associated with diverse cellular activities (AAA) family of proteins known to be involved diverse range of cellular processess including Golgi, endoplasmic reticulum, and nuclear membrane reassembly , protein degradation, cell cycle control, autophagy, and mitochondrial quality control. p97 is widely distributed throughout the cell and accounts for about 1% of total cellular protein. Previous studies have suggested that p97 acts as an ubiquitin-selective chaperone with the primary function of unfolding proteins and disassembling protein complexes. Ubiquitin molecules are small proteins that mark certain proteins for destruction by proteolytic enzymes known as proteosomes. As an ATPase, p97 works by using chemical energy generated from ATP hydrolysis and converting it into mechanical force which changes the conformation of targeted proteins and protein complexes. The wide range of processes p97 is involved with each having distinct binding patterns and varying cofactor proteins. The role of p97 and the mechanisms for many of these processes are still being investigated. This review will summarize studies on p97's roles in muscle atrophy, mitochondrial membrane protein turnover, and the effects of p97 inhibition.

P97 and Muscle Atropy

Piccirillo and Goldberg of Harvard Medical School's Department of Cell Biology published an article in the July 2012 edition of the European Molecular Biology Organization (EMBO) Journal studying if p97 participates in the rapid degradation of myofibrillar proteins during muscle atrophy. The balance of overall rates of protein synthesis and degradation determine the size and functional capacity of muscle. Various diseases, such as diabetes and cancer, fasting, and inactivity all cause skeletal muscle to lose mass and contractile capacity. It is believed that this muscle atrophy is related to the ubiquitin-proteasome system, of which p97 is a key compenent.

This study focuses on whether p97 and its cofactors have a critical role in the breakdown of the myofibrillar apparatus during rapid muscle wasting induced by denervation or fasting. They first tested to see if inhibiting p97 would reduce muscle atrophy by comparing the effects of expressing wild type p97 (WTp97) and a mutant p97K524A, a dominant negative inhibitor (DNp97) which lacks affinity to certain protein receptors. To identify the two p97 types in the muscle fiber of mice, they were fused with green florescent protein (GFP). The WT, DNp97, and GFP only control DNA plasmids (20μg) were electroporated bilatterally into the Tibialis Anterior (TA) muscles in the hindlimbs of adult mice. During this time, the sciatic nerve was cut and the downstream muscles were allowed to atrophy for nine days for the denervation study. After nine days the

muscles were collected and cryosectioned, and flouresence microscopy was used to view the transfected fibers.

The results, measuring the cross-sectional area of the TA muscles, showed little or no effect from the GFP control or the WTp97 in the denervated muscles, however, the electroporation of the DN to inhibit p97 showed a reduction of atrophy of roughly 60%. These numbers were actually almost exactly the same as the cross sectional areas of innervated TA muscles that demonstrated that p97 inhibition seemed to prevent muscle atrophy by deneveration completely.

The fasting study had a fed (control) and fasted group which had the same initial body weights prior to the experiement and followed a similar process of injecting plasmids into the mice. The fasting group was deprived of food for 48 hours to simulate muscle atrophy induced by starvation and then the muscles were collected. The fasting mice injected with GFP and the mice with WTp97 showed muscle fibers 33% smaller than the fed mice. The fasting mice injected with DNp97 showed a 70% increase in median cross-sectional area over the fed mice. This came as a surprise in that the inhibition of p97 not only inhibited muslce atrophy but actually promoted hypertrophy, or muscle growth.

Another test was performed to see if increasing the amount of WTp97 increases the rate of muscle atrophy. TA muscles of fed mice were electroporated with plasmids of WTp97 or DNp97 fused with GFP. After seven days the cross sectional area of TA fibers expressing WTp97 were similar to surrounding fibers which were not electrporated with WTp97; there was no change. The fibers inhibited with DNp97 showed a 60% increase in median fiber area. This showed that increasing the amount of WTp97 does not inhibit muscle growth, while inhibiting p97 induces growth.

Since it is not possible to measure the amounts of protein synthesis and degradation occuring inside the mice, a test was performed with cultured cells to determine whether the inhibition of p97 by DNp97 promotes fiber growth in myotubes as well as adult muscles. Instead of transfecting muscle fibers in mice, C2C12 myotubes were infected with adenoviruses encoding GFP, WTp97, or DNp97 instead. As the cultures were grown over a 48 hour period, the levels of each protein increased. The DNp97 infected cultures showed increases in cell diameter while the WTp97 and the GFP infected cultures showed no change. These results were simliar to those seen in the experiments conducted with TA muscle fibers in mice.

It was then examined if the DNp97 results were due to an overall increase in protein synthesis or a reduction in protein degradation. ^{3}H-tyrosine was incorporated into total cell proteins and the rates were measured with no difference observed upon overexpression GFP, WTp97, or DNp97. No increases in phosphoylation of AKT (Protein Kinase B) or the mammalian target of rapamycin (mTOR) protein effecters were found downstream either. Thus it was determined that DNp97 induced growth by inhibiting overall proteolysis or degradation without increasing rates of synthesis.

P97 is Essential for Outer Mitochondrial Membrane Protein Turnover

The ubiquitin-proteasome system also plays a role in the regulation cycle of outer mitochondrial membrane (OMM) associated proteins. A study conducted by Xu et al. showed that p97 is required for the proteosomal degradation of two OMM proteins, Mcl1 and Mfn1, which are unrealated and have short half-lives. In the mitochondria the OMM is the barrier between the mitochondria and the cytoplasm. It performs functions that are vital to the function of mitochondria such as regulating metabolism and apoptosis. Therefore, the maintainance of the regulation of the OMM is essential. It is known that the degradation of certain OMM associated proteins such as Mcl1, an anti-apoptotic protein, and Mfn1, a mediator of mitochondrial fusion, are associated with the ubiquitin-proteasome system, and hence, p97.

The study addresses whether or not p97 regulates the turnover of OMM associated proteins. First, an array of OMM associated proteins was analyzed for stability and ubiquitin-proteosome dependence. Mcl1 and Mfn1 both have short half-lives compared to a mitochondrion and were both stabalized by the proteosome inhibitor, MG132, and degraded when the protein synthesis inhibitor cycloheximide (CHX) was present. Other OMM associated proteins, such as Tom20, were used as controls and did not change with exposure to CHX or MG132. An experiement using HeLa cells, a common immortal cell-line frequently used in scientific research, treated with CHX or MG132 for 0, 5, or 10 hours followed by Western blotting confirmed the results.

HeLa cells were then transfected with three different p97 shRNAi (small hairpin RNA interference) constructs, which are used as gene silencers or inhibitors by targeting gene expression on RNA, or with a GFP shRNAi construct which was used as a control. Mfn1, Mcl1, p97, and Tom20 were each exposed to the four cell cultures then Western blots were taken to measure protein levels. The results showed that the effects of the p97 inhibitors closely resembled the MG132 treated cells in that Mfn1 and Mcl1 both showed slight protein level growth when exposed to the p97 inhibited cell cultures and degradation when exposed to the uninhibited GFP control.

Another experiement showed that a mutation to p97 inhibits proteasomal degradation of Mcl1. Control RNAi and p97 RNAi cells were treated with CHX for varying

amounts of time then analyzed by Western blotting. The results showed almost no p97 degradation in CHX treated p97 RNAi cells and complete degradation with the CHX treated control RNAi which confirmed the notion that p97 helps regulate Mcl1 turnover.

A similar test using a dominant negative p97 ATPase mutant, $p97^{QQ}$, was done to see if p97 activity is required for Mcl1 degradation. $p97^{QQ}$ has been shown to inhibit the retrotranslocation of various ER proteins from the ER membrane to the cytoplasm, which helps stabalize the proteins. The results show that the inhibition of p97 by $p97^{QQ}$ expression resulted in an increase in Mcl1 levels which confirmed that the regulation of degradation of Mcl1 does require p97.

It was found that p97 inhibition stabalizes both Mcl1 and Mfn1 as well as hinders their movement from the OMM to the cytosol. This was determined by testing to see where the p97 acts in relation to the proteosome, upstream or downstream. Testing involving p97 and $p97^{QQ}$ treated with the proteosome inhibitor MG132 followed by Western blot analysis showed that the proteasome function was not affected by the inhibition of p97, therefore, p97 must act upstream of the proteosome.

Using DBeQ as a Reversible Inhibitor of p97

A study conducted by Chou et al. identified N^2,N^4-dibenzylquinazoline-2,4,diamine (DBeQ) as a selective, potent, reversible, and ATP-competitive inhibitor of p97 that has potential to be used to target and fight human disease, namely cancer. Previous studies had used mutant dominant negative versions of p97 or shRNAi to inhibit its function but these provided no method to rapidly shut off the inhibition. The discovery of DBeQ as a reversible p97 inhibitor will allow for greater research into the function of p97 in cells.

DBeQ emerged as a selective and potent p97 inhibitor after using an assay of p97 screened against two libraries of known p97 inhibitors from the Maybridge Hitfinder Collection and the National Institutes of Heath Molecular Libraries Small Molecule Repository. 925 hits were found which were then screened further down to 10 compounds by amount of inhibition of p97 and molecule size. These 10 compounds were then evaluated for their selectivity towrads p97 and their ability to inhibit degradation of p97 independent proteasome substrates. Western blot analysis of the select substrates was performed against each compound with DBeQ being the least potent towards the substrates while still inhibiting p97.

Discussion

From the studies conducted by Piccirillo and Goldberg, it was shown that p97 does play an essential role in muscle atrophy. Yet, overexpression of p97 does not lead to a induced rate of muslce wasting. Even though p97 is necessary for atrophy, its level is not a rate-limiting factor for proteolysis. Inhibiting expression of p97 leads inhbits muscle atrophy and can even cause muscle hypretrophy. This discovery will most likely be further studied and may be beneficial in the fight of a variety of diseases.

The data collected in these experiments by Xu et al. help scientists better understand how p97 is linked to retrotranslocation of OMM associated proteins and the OMM-associated degradation pathway. p97 inhibition was found to stabalize both Mcl1 and Mfn1 proteins of the ubiquitin-proteasome system as well as hinder their movement from the OMM to the cytosol. This proves that unhindered p97 will cause degradation or translocation of these proteins in the mitochondria. More work needs to be done to see how which cofactors are needed for the mitochondrial function of p97 and clarification is needed on the extent of the involvement of p97 in the regulation of mitochondrial proteostasis.

With the indentification of DBeQ as a potent and selective p97 inhibitor by Chou et al, it may now be easier to idenify ubiquiton-proteosome system substrates whose degradation is dependent on p97. It is believed DBeQ and the inhibition of p97 may become a succesful strategy for cancer chemotherapy. DBeQ has the ability to induce rapid cell death in tumors be inhibiting p97 activity. Further studies need to be conducted to better understand the mechanism by which DBeQ inhibits p97 and how the inhibition results in apoptosis in select cells which can be targeted towards cancerous cultures.

Table 1: Inhibitors of p97

INHIBITOR	EFFECT
mutant p97 (e.g. p97K524A)	dominant negative inhibitor (DNp97) competitively inhbits p97; lacks affinity for certain protein receptors which may effect ubiquination
p97 shRNAi	targets p97 gene expression on RNA
DBeQ	reversible inhibitor of p97, can turn inhibition on or off

References

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