The Effects of NAADP binding proteins, JPT2 and LMS12 on the levels of autophagy

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Introduction

- Autophagy is a self-digestion process in cells, recycling old proteins into amino acids to build new organelles. It is upregulated in nutrient scarcity, aiding in cell repair and survival.[1]

- Therefore, researching how to regulate autophagy has significant therapeutic potential for conditions caused by cell stress, like Parkinson's.

- The reduced expression of the two autophagic markers p62 and LC3 indicates high levels of autophagy.[2]

- Nicotinic acid adenine dinucleotide phosphate, NAADP, is a second messenger that releases Ca2+ by activating ion channels known as two-pore channels (TPCs), on lysosomes, a cellular organelle.[3]

- NAADP activates TPCs indirectly by binding to a NAADP binding protein, like JPT2 and LMS12, associated with the TPC complex.

- Lysosomes are the final destination of the autophagy pathway and there are studies that provide evidence that NAADP-evoked Ca2+ signals mediated by TPCs regulate autophagy.

- My aim was to gain a deeper understanding of the link between the role of calcium signalling and autophagy by researching the effect of the presence/absence of JPT2 and LMS12 on the level of autophagy.

- Two U2OS samples were used: Mock cells, or 'wild-type cells', and double knock-out cells (DKO), where the genes for JPT2 and LMS12 were removed using gene editing.

Figure 1. Autophagy Pathway[4]

Methodology:

Treatments:

- Two drugs were used to influence autophagy: Torin-1, an inducer; and Bafilomycin A1, an inhibitor.

- Torin-1 stimulates the formation of autophagosomes, and bafilomycin-1 prevents the formation of the autolysosome.[5]

- With both treatments, a increase of autophagosomes was expected, indicated by a high expression of both LC3-II and p62.

- Comparing control with Baf A1: **Basal autophagy**

- Comparing Torin with Torin + Baf A1: **Torin-induced autophagy**

- 2 sets, one mock and one DKO of 4 samples were used per experiment: 1) Control (no treatment, 2) Baf A1, 3) Torin-1, 4) Baf A1 and Torin -1.

Western Blotting:

1. The samples were lysed to separate the non-soluble components and proteins, then denatured via heating.

2. The samples and a protein ladder (for tracking migration) were loaded into wells and separated by size via gel electrophoresis, with smaller proteins migrating farther. Gels were then transferred to a membrane.

3. The membranes were blocked in milk to prevent nonspecific antibody binding.

4. Primary antibodies were added to bind to target proteins in a one-hour incubation and TBS-T washing.

5. Secondary antibodies, linked to signal molecules like chemiluminescent markers, were added to visualise the bound primary antibodies, thus indicating the target protein's presence. Membranes underwent an hour of incubation and TBS-T washing.

6. The membranes were coated with substrate and then imaged in a Chemidoc machine, capturing both chemiluminescent and colorimetric images for sample and protein ladder detection.

7. Steps 1-6 are repeated for actin, which is used as a loading control for Western Blot to normalize the levels of protein detected by confirming that protein loading is the same across the gel.

Results

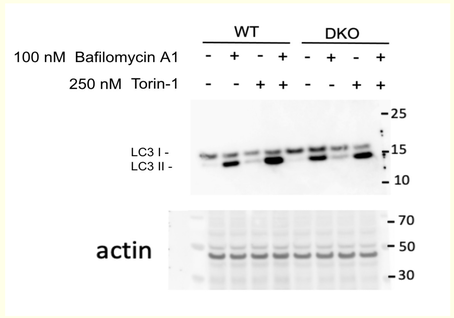

Figure 2. Western-blot analysis of wild type cells and DKO cells expressing LC3 after 3-hour treatments of Baf A1 and Torin-1. Actin used as a loading control.

- LC3-II is used as an autophagy marker for analysis because it is the form of LC3 that is recruited to the autophagosome membrane.

- The darker the colour, the higher the level of that protein is being expressed in that sample.

- The number on the Y-axis indicate kDa, kilodaltons, a unit of measurement of the molecular masses of proteins.

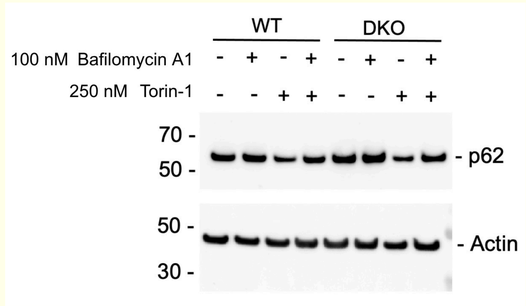

Figure 2. Western-blot analysis of wild type cells and DKO cells expressing p62 after 3-hour treatments of Baf A1 and Torin-1. Actin used as a loading control.

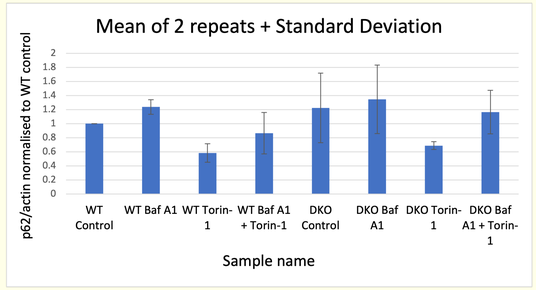

Figure 4 – A chart showing the relative amounts of p62 protein in each sample divided by the relative amounts of actin normalised to the wild-type control sample.

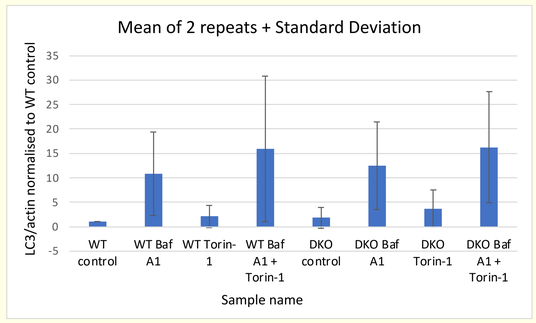

Figure 4 – A chart showing the relative amounts of LC3 protein in each sample divided by the relative amounts of actin normalised to the wild-type control sample.

Conclusion:

- For both p62 and LC3, there was no significant difference in autophagy between WT and DKO.

Discussion:

- There are a few possible limitations that could have explained the reason why the results were not as expected.

- Firstly, the protein quantification is only semi-quantitative, giving less precise outcomes. The correlation between signal strength on the blot and protein quantity is not consistently linear. Therefore, there is not an absolute quantification of autophagic flux.

- There was a small number of repeats, giving less reliable results, and large standard deviation.

- Some unclear blots were produced, which could have decreased the accuracy of the results.

- Bafilomycin A1 may have some unwanted effects since it interacts with the V-ATPase proton pump on the lysosome, therefore Bafilomycin A1 may interfere with the NAADP activation of the TPCs. This may indirectly affect the levels of the NAADP binding proteins, JPT2 and LMS12.

- LC3 and p62 levels can change due to unrelated reasons apart from autophagy. For example, the levels of LC3 can change due to the inhibition of autophagosome-lysosome fusion or the impairment of lysosomal degradation, not just autophagy.

Future steps:

- Utilise electron microscopy: which offers direct visualization of autophagic structures, resulting in the absolute quantification of autophagosomes and autolysosomes to assess autophagic activity.

- Utilise combination inhibitors that affect proteases rather than the channels in the autophagy pathway.

- Include more autophagy markers, like Beclin-1, ATG5, and ATG7.

- Perform a Western-Blot analysis for other TPC related compounds like TPC2-A1-N, which mediates NAADP-like TPC2 activation, inducing a calcium influx.

References:

1. Yu, Li, Yu Chen, and Sharon A. Tooze. 2018. "Autophagy Pathway: Cellular and Molecular Mechanisms." Autophagy 14 (2): 207-215.

2. Tanida, Ichiro, Tetsuhiro Ueno, and Eiichi Kominami. 2008. "LC3 and Autophagy." In Autophagosome and Phagosome, edited by Vojo Deretic, 77-88. Tokyo: Humana Press.

3. Marchant, Jonathan S., et al. 2022. "NAADP-binding Proteins Find Their Identity." Trends in Biochemical Sciences 47 (3): 235-249.

4. Agrotis, Alexander. "Figure 1." Created for use in UCL.

5. Andersson, Anna-Maria, et al. 2016. "Autophagy Induction Targeting mTORC1 Enhances Mycobacterium tuberculosis Replication in HIV Co-infected Human Macrophages." Scientific Reports 6.

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