

Interpretation of bafilomycin, pH neutralizing or protease inhibitor treatments in autophagic flux experiments

Novel considerations

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Recent publications showed that the kinase MTOR localizes to lysosomes and its activation depends on amino acids inside the lysosomal lumen, implying that autophagic protein degradation is a positive regulator of MTOR in this setting. Since decreased MTOR activity results in autophagy induction, drug treatments that block autolysosomal degradation (a commonly used technique to estimate autophagic flux) may actually interfere not only with lysosomal breakdown, but also increase autophagosome generation through impaired MTOR signaling.

Autophagy ensures the autophagosome-mediated transport of cytoplasmic material to lysosomes for degradation and re-use. By now it has become widely accepted that analyzing the number of autophagy-related structures alone is an inadequate measure of autophagic degradation activity (flux), as increased autophagosome numbers can reflect both their accelerated generation or decreased clearance. Similarly, the number and size of autolysosomes depend on both 'input': material delivered by autophagosomes, and 'output': material degraded and recycled back to the cytoplasm for re-use. The levels of activated, lipidated Atg8/MAP1LC3 family proteins (mostly referred to as Atg8-II or LC3-II) are increased during autophagy induction which can be easily followed in western blot experiments, but since Atg8 molecules linked to the inner autophagosomal membrane are subject to degradation in autolysosomes, their levels also depend on both activation (i.e.

autophagy induction) and degradation (i.e. breakdown in autolysosomes).¹

One popular method for the estimation of autophagic flux is to carry out experiments in the absence and presence of lysosomal protease inhibitors (such as leupeptin), intralysosomal pH neutralizing agents (such as chloroquine), or drugs that inhibit the vacuolar type H⁺-ATPase (v-ATPase) complex necessary for lysosomal acidification (such as bafilomycin A₁). These treatments block autophagic degradation at a step downstream of autophagosome formation, allowing one to make a direct comparison of autophagosome numbers or lipidated Atg8/LC3 levels with experiments done without using these drugs.¹

MTOR (mechanistic target of rapamycin) is a serine/threonine kinase that integrates multiple signals such as growth factor abundance with intracellular amino acid and ATP levels to coordinate cell growth and autophagy. MTOR promotes translation in a number of ways including the activation of RPS6KB1/2 (ribosomal protein S6 kinase, 70 kDa, polypeptide 1/2) and inhibition of EIF4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) and blocks autophagy by inhibiting the Atg1/ULK1 (unc-51-like kinase 1) kinase complex.² MTOR is rapidly inactivated upon starvation that results in autophagy induction typically peaking at 2–4 h depending on the experimental system. Recent papers showed that autophagy is required for the re-activation of MTOR and repression of autophagy during prolonged starvation, and that amino acids inside lysosomes

Keywords: Atg8, autophagy, bafilomycin A₁, chloroquine, LC3, leupeptin, lysosome, MTOR, TOR, v-ATPase

Submitted: 06/25/12

Revised: 07/17/12

Accepted: 07/19/12

<http://dx.doi.org/10.4161/auto.21544>

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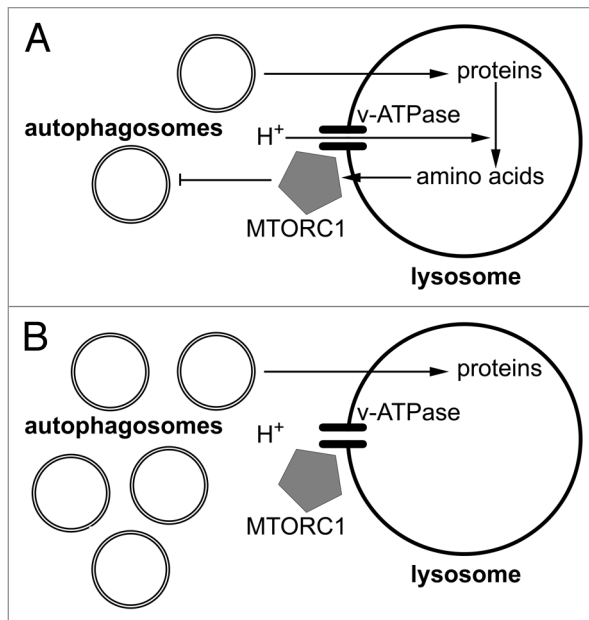


Figure 1. Schematic representation of the connection between autophagy and MTOR signaling mediated by lysosomes. (A) Induction of autophagy results in autophagosome-mediated delivery of proteins to lysosomes. The v-ATPase complex ensures optimal acidic pH for lysosomal proteases that break down proteins into amino acids, which in turn activate MTOR complex 1 associated with the v-ATPase complex. MTORC1 activation then results in feedback inhibition of autophagosome generation. (B) Inhibition of the v-ATPase proton pump, neutralization of lysosomal acidity or block of lysosomal proteases decreases autophagic protein degradation and intralysosomal amino acid levels, likely leading to MTORC1 inactivation and increased formation of autophagosomes.

are necessary for MTOR activity.^{3,4} Also, the regulator complex required for amino acid-induced MTOR activation recruits MTOR complex 1 to the v-ATPase complex and ensures the localization of active MTOR on lysosomes (Fig. 1A).⁵

In light of these new results, we may need to re-evaluate the use of inhibitors that block lysosomal protein degradation for autophagic flux. Bafilomycin, chloroquine and protease inhibitor treatments are very likely to interfere with MTOR activity, potentially resulting in further acceleration of autophagosome formation unless MTOR is already completely inactive in the experimental setting (Fig. 1B).

Therefore, estimating MTOR activity by looking at phospho-RPS6KB1/2 and phospho-EIF4EBP1 levels should be an essential part of flux assays that involve these drug treatments. Along these lines, finding of a drug or genetic mutation that specifically blocks only the fusion of autophagosomes with endosomes or lysosomes and not other vesicle fusion events would provide a useful tool for the autophagy community.

Acknowledgments

Work in the author's lab is funded by the Wellcome Trust (087518/Z/08/Z) and the Hungarian Scientific Research Fund (OTKA K83509).

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