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Effects of mTORC1 inhibition on proteasome activity and levels

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ABSTRACT

The mechanistic target of rapamycin (mTOR) regulates numerous extracellular and intracellular signals involved in the maintenance of cellular homeostasis and cell growth. mTOR also functions as an endogenous inhibitor of autophagy. Under nutrient-rich conditions, mTOR complex 1 (mTORC1) phosphorylates the ULK1 complex, preventing its activation and subsequent autophagosome formation, while inhibition of mTORC1 using either rapamycin or nutrient deprivation induces autophagy. Autophagy and proteasomal proteolysis provide amino acids necessary for protein translation. Although the connection between mTORC1 and autophagy is well characterized, the association of mTORC1 inhibition with proteasome biogenesis and activity has not been fully elucidated yet. Proteasomes are long-lived cellular organelles. Their spatiotemporal rather than homeostatic regulation could be another adaptive cellular mechanism to respond to starvation. Here, we reviewed several published reports and the latest research from our group to examine the connection between mTORC1 and proteasome. We have also investigated and described the effect of mTORC1 inhibition on proteasome activity using purified proteasomes. Since mTORC1 inhibitors are currently evaluated as treatments for several human diseases, a better understanding of the link between mTORC1 activity and proteasome function is of utmost importance.

INTRODUCTION

The mechanistic (formerly mammalian) target of rapamycin (mTOR) is an atypical serine/threonine protein kinase of the phosphatidylinositol 3-kinase-related kinase superfamily. mTOR coordinates several signaling networks that promote anabolic processes and control cellular and organismal growth (1, 2). In mammals, there are two distinct, but potentially complementary, catalytic complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (3). Numerous extracellular stimuli such as growth factors and mitogens can initiate mTORC1 activation by inhibiting tuberous sclerosis complex (TSC), which functions as another pleiotropic hub integrating subcellular energy and amino acid availability (4). Besides growth factors, cellular amino acid pool can also activate mTORC1, which affects global translation not only as a rate-limiting reactant, but also as an upstream regulator of signaling pathways.

In the presence of amino acids, mTORC1 is activated, leading to the activation of downstream effectors to utilize amino acids via upregulation of global translation (5, 6). mTORC2 is not activated by amino acids (7). Under normal conditions, the ubiquitin-proteasome system (UPS) is the primary catabolic system that degrades proteins and provides cells with amino acids for new protein synthesis (8). If the amino acid supply is limited, bulk autophagy, another cellular degradation system, is induced to provide additional anabolic intermediates (9). For example, the inhibition of UPS rapidly depletes available amino acids, leading to compensatory induction of autophagy to restore cellular proteolytic capacity, while the activation of proteasome results in the suppression of autophagy (10-14). These two pathways appear to be connected via a negative feedback mechanism. As a whole, they coordinate their functions to maintain the cellular amino acid pool.

By inhibiting the early stage of cellular autophagy flux, the mTOR pathway can

prevent futile cycles of continuous proteolysis and translation. mTORC1 can directly phosphorylate and suppress autophagy-initiating kinase ULK1 (15, 16). Inhibition of mTOR mimics nutrient-depleted conditions, subsequently inducing autophagy. mTOR inhibitors such as rapamycin and Torin1 are widely used both *in vitro* and *in vivo* to decipher the role of mTOR in diverse disease models (17, 18). Rapamycin is a natural compound with potent antitumor and immunosuppressive properties (19). It forms a complex with FK506-binding protein 12 (FKBP12) and interacts with mTORC1, but not mTORC2, to inhibit its activity. Torin1 is a synthetic ATP-competitive mTOR inhibitor that can potently block the activation of both mTORC1 and mTORC2 (20).

Although the link between mTOR and autophagy regulation has been established, the biochemical connection between the mTOR pathway and proteasomal protein degradation remains unclear. Furthermore, even though the regulation of 26S proteasome synthesis is well studied, much less is known about the role of mTOR in this process. In this review, we will focus on the effect of mTOR inhibition on proteasome homeostasis and overall proteolysis without providing a comprehensive synopsis of signaling networks. We will summarize key findings from three essential articles (21-23) as well as the latest research from our group.

CASE #1: INHIBITION OF mTORC1 SUPPRESSES PROTEASOME BIOGENESIS AND GLOBAL PROTEASOMAL DEGRADATION

Only a few studies so far have investigated the connection between the mTOR pathway and 26S proteasome. Zhang *et al.* have reported that in *TSC1*- or *TSC2*-deficient mouse embryonic fibroblasts (MEFs), activation of mTORC1 can upregulate the biogenesis of proteasome subunits and other auxiliary proteins and increase cellular proteolysis rates in

serum-starved (i.e., autophagy-induced) cells (21). Furthermore, they found that inhibition of mTORC1 activity with 20 nM rapamycin for 16 h reduced cellular proteasome levels and global proteolysis capacity (21). In response to mTORC1 activation, nuclear factor erythroid-derived 2-related factor 1 (NRF1/NFE2L1) induced the expression of proteasome subunit genes (21). NRF1 is a well-known key transcription factor involved in the regulation of mammalian proteasome gene expression. Protein levels of this transcription factor are maintained at a low basal level via endoplasmic reticulum (ER)-associated protein degradation (24). In response to proteasome inhibition, NRF1 can escape degradation and upregulate the expression of all 33 proteasome subunit genes and their assembly factors (25).

Zhang *et al.* (26) also reported that the observed proteolytic rates were primarily due to proteasomal proteolysis, although they did not directly measure the effect of autophagic flux. They showed that chloroquine, a lysosomal inhibitor, had no effect on the rate of proteolysis in cells with increased mTORC1 activity, while bortezomib, a proteasomal inhibitor, effectively abrogated this effect. In addition, even in autophagy-deficient *Atg7^{-/-}* MEFs, a long-term rapamycin treatment significantly decreased the protein degradation rate. Physiological activation of mTORC1 in mouse liver by re-feeding after fasting induced *NRF1* and the expression of other proteasome-related genes (21). Collectively, these results suggest the existence of a complex network responsible for the maintenance of cellular amino acid pool. mTORC1 activation induces amino acid-utilizing anabolic process and upregulates proteasome level/activity (to produce amino acids) at the same time, while downregulating autophagy. NRF1 is the key factor connecting these systems. These results also point to the existence of a crosstalk between the UPS and autophagy. That is, a negative-feedback loop allows cells to maintain a proper cellular amino acid pool.

CASE #2: INHIBITION OF mTORC1 INDUCES GLOBAL PROTEASOMAL DEGRADATION WITHOUT AFFECTING PROTEASOME BIOGENESIS

The coupled pathways presented in Case #1 (abundant amino acids → mTORC1 activation → 1) ribosome/translation factor, 2) NRF1 → 1) global translation, 2) proteasome biogenesis → 1) larger proteome, 2) proteasome activity → adequate amino acid pool and protein quality) as a possible adaptive response mechanism have been challenged by many groups. Zhao *et al.* (22) have reported that rapamycin or Torin1-induced mTORC1 inhibition in complete medium can enhance global proteolysis, which is opposite to the observations presented by Zhang *et al.* (21). Only long-lived proteins (pulse-labeled for 20 h), but not short-lived proteins (labeled for 20 min), were subjected to stimulated proteolysis. This phenomenon was not linked to changes in proteasome gene transcription or synthesis after mTORC1 inhibition (note that NRF1 activation was not measured) (22). Such discrepancy could be due to differences in culture media, inhibitor concentrations, treatment durations, or pulse-chase methods measuring global protein degradation rate. Zhao *et al.* (27) have speculated that longer pulse labeling with ^3H -Phe could avoid the reincorporation of unlabeled radioisotopes during chase periods.

Zhao *et al.* (22) have observed that mTOR inhibitors can stimulate both UPS- and autophagy-dependent proteolysis. They hypothesized that higher proteasomal degradation rates were mediated by elevated polyubiquitylation. In response to inhibition of mTOR activity, long-lived (and rarely polyubiquitylated) proteins became rapidly (less than 30 min) ubiquitylated with Lys48-linked chains and subsequently degraded by the 26S proteasome. Their study implies that the cellular UPS system operates at less than its full capacity because of mTORC1 activity. mTOR inhibition could benefit cells under stress conditions by not only delaying their growth, but also enhancing proteolytic capacity. However, several questions

remain unanswered, including the following: 1) which mechanisms are responsible for increased global polyubiquitylation after mTOR inhibition? and 2) why both UPS and autophagy need to be activated in response to relatively mild metabolic stress. Notably, the same group has reported that 26S proteasomes could be directly phosphorylated by protein kinase A (PKA), boosting proteasomal proteolysis of short-lived proteins (28, 29).

OTHER EXAMPLES OF mTORC1 INHIBITION AND PROTEASOME HOMEOSTASIS/ACTIVITY

It has been reported that selective inhibition (using 0.2 $\mu\text{g/mL}$ rapamycin for 3 h) of TORC1 in yeast can facilitate 19S proteasome assembly and increase cellular proteasome levels/activity more than 2-fold (30). Treatment of yeast with ER stress inducers such as tunicamycin can result in similar outcomes (30). In general, ER stress antagonizes cellular anabolism. However, it can either enhance or suppress mTORC1 signaling (23). The coordinated regulation between mTOR and ER stress is not fully understood yet. Rousseau and Bertolotti (30, 31) have reported that the increased proteasome abundance after TORC1 inhibition is mediated by the mitogen-activated protein kinase Mpk1/ERK5 and Adc17 which is an inducible chaperone for proteasome assembly. This might contribute to the rapid and reversible elimination of misfolded proteins during ER stress.

We have also investigated the effect of mTORC1 inhibition on proteasome activity and function using purified proteasomes from HEK293 cells (32-34). We found that mTORC1 inhibition with 250 nM Torin1 did not significantly affect proteasome content or direct phosphorylation on proteasome (Figs. 1a – 1c). When purified proteasomes were analyzed using non-denaturing (native) polyacrylamide gel electrophoresis and subsequent in-gel

activity analysis, Torin1-treated proteasomes showed structural integrity and activity similar to control 26S proteasomes (Fig. 1d). We did not find any significant differences in chymotrypsin-like $\beta 5$ proteasome activity (measured by suc-LLCY-AMC hydrolysis) or other catalytic activities (such as caspase-like $\beta 1$ and trypsin-like $\beta 2$ activities) either in whole-cell lysates upon Torin1 treatment (Fig. 1e and data not shown). Taken together, these results indicated that inhibition of mTORC1 did not appear to rapidly alter cellular proteasome levels or activity in HEK293 cells.

CONCLUSION

Currently, reports investigating effects of mTORC1 inhibition on the synthesis of 26S proteasomes have presented contradictory results. Cells have numerous adaptive strategies to cope with an increased number of target substrates either at the translational level or the posttranslational level. Considering that the proteasome is long-lived (half-life $> \sim 10$ days in mammals (35-37)) and present in excess (more than 1% of yeast proteome (38)), its spatiotemporal regulation could be more adaptable to efficient proteolytic responses to stress than homeostatic regulation. For example, instead of energy-costly degradation and synthesis, proteasomes could be sequestered into cytoplasmic granules during quiescence, nuclear foci under hypertonic stress, or aggresomes when catalytically inhibited (39-41). The spatiotemporal sequestration of proteasomes appears to be mediated by reversible liquid-liquid or liquid-gel phase separation. Proteasomes in liquid droplets can recover to be a functional enzyme in the absence of cellular stress, while proteasomes in less soluble aggregates might have already lost their structural integrity and thus they are destined for autophagic degradation.

Despite tremendous progress in mTOR biology (an anabolic process), many questions

regarding its role in the UPS (a catabolic process) remain to be addressed. The molecular mechanisms regulated by mTOR as an amino acid sensor and cellular autophagy suppressor have been thoroughly investigated. However, our understanding of the role of the proteasome in the mTOR signaling cascade is far from complete. It is essential to confirm the effect of mTORC1 inhibition on 26S proteasome biogenesis by independent groups using standardized protocols. In addition, quantification of cellular amino acid pool, NRF1 activation, and polyubiquitin linkages in response to either chronic or acute mTORC1 inhibition is necessary. These results will provide mechanistic clues to clarify the remaining pathways of this complex signaling circuit. Considering that dysregulation of cellular amino acid pool is implicated in a diverse array of human diseases, the ability to regulate mTORC1 and proteasome activity levels could further extend therapeutic applications of mTORC1 inhibitors.

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FIGURE LEGEND**Figure 1. Characterization of purified 26S proteasome after treatment with Torin1.**

(a, b) Effect of mTORC1 inhibitor Torin1 on proteasomes. HEK293 cells were treated with 250 nM Torin1 for indicated time periods. Whole-cell lysates (a) and purified proteasomes (b) were collected and separated on denaturing SDS-PAGE. (c) Comparison of phosphorylation of purified proteasomes using pan-phosphorylation antibodies (61-8300, Invitrogen, 1.0 µg/ml). (d, e) Effect of Torin1 treatment on proteasome activity. Proteins in whole HEK293 lysates were separated on non-denaturing (native) PAGE, followed by in-gel suc-LLVY-AMC hydrolysis or immunoblotting against the 20S subunit, PSMA4, to visualize proteasomes (d). Protein samples (10 µg) were subjected to suc-LLVY-AMC (12.5 µM) hydrolysis to estimate proteasome activity (e).

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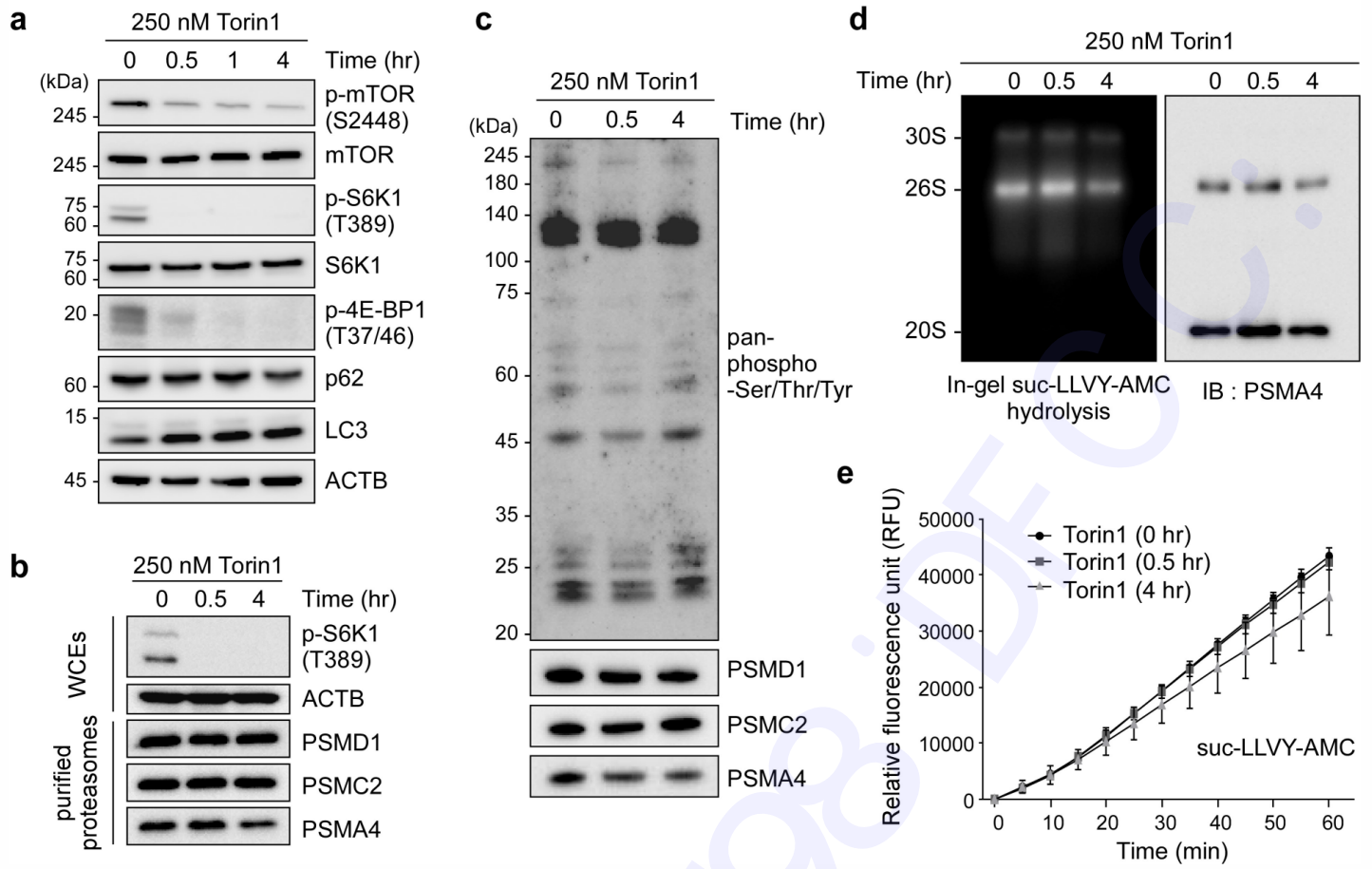


Fig. 1.