Intracellular Ca\textsuperscript{2+} signaling: A novel player in the canonical mTOR-controlled autophagy pathway

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Abbreviations: AMPK, AMP-activated kinase; Arg, Autophagy protein; ATP, adenosine triphosphate; BAPTA(-AM), 1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid (-acetoxymethyl ester); Bcl-2, B cell CLL/lymphoma 2; CaMKI, Calmodulin kinase I; CaMKKB, Calmodulin kinase β; Dox, doxycycline; EGTA, ethylene glycol tetraacetic acid; ER, Endoplasmic reticulum; ERK, Extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ins(1,4,5)P\textsubscript{3}R, Inositol 1,4,5-trisphosphate receptor; LC3, Microtubule-associated protein 1 light chain 3; MEF, Mouse embryonic fibroblast; mTOR, Mammalian target of rapamycin; NAADP, Nicotinic acid adenine dinucleotide phosphate; PKC\textsubscript{θ}, Protein kinase C θ; SERCA, Sarco-/endoplasmic reticulum Ca\textsuperscript{2+} ATPase; ULK1, Unc-51-like kinase 1; WIPI-1, WD repeat domain phosphoinositide-interacting protein 1

Intracellular Ca\textsuperscript{2+} signaling is essential for the upregulation of the canonical mTOR-controlled autophagy pathway triggered by rapamycin or by nutrient deprivation. Moreover, modifications in the Ca\textsuperscript{2+}-signaling machinery coincide with autophagy stimulation. This results in enhanced intracellular Ca\textsuperscript{2+} signaling essential for driving the autophagy process. Yet, the mechanisms upstream (the players causing the changes in Ca\textsuperscript{2+} signaling) and downstream (the targets of the altered Ca\textsuperscript{2+} signals) of this Ca\textsuperscript{2+}-dependent autophagy pathway remain elusive. Here, we speculate about these mechanisms based on our current knowledge.

To Have a Look Upstream: The Culprit(s) of the Changes in Ca\textsuperscript{2+} Signaling

The role of Ca\textsuperscript{2+} signaling in autophagy regulation was met with a great deal of controversy in the past years, with reports suggesting inhibitory, as well as stimulatory, effects of Ca\textsuperscript{2+} on autophagy.\textsuperscript{1} We recently investigated the role of intracellular Ca\textsuperscript{2+} homeostasis/signaling in canonical mTOR-controlled autophagy upon rapamycin treatment.\textsuperscript{2} Rapamycin activates autophagy via the inhibition of mTOR, which is regarded as a key regulator of autophagy, leading to its stimulation through a canonical pathway, involving downstream of mTOR, the ULK1 complex, the PI3K complex III (including Beclin 1), Atg5, Atg7 and LC3. Rapamycin treatment of HeLa and MEF cells stimulated autophagy in a concentration- and time-dependent manner. Interestingly, the upregulation of autophagy correlated with an increase in the ER Ca\textsuperscript{2+} content and increased Ins(1,4,5)P\textsubscript{3}R-mediated Ca\textsuperscript{2+} signaling. Buffering the intracellular Ca\textsuperscript{2+} with the cell-permeable chelator BAPTA-AM abolished rapamycin-induced autophagy, indicating that intracellular Ca\textsuperscript{2+} signaling is required for activation of the canonical autophagy pathway. This study followed up on a previous study investigating the role of Ca\textsuperscript{2+} signaling in nutrient starvation-induced autophagy.\textsuperscript{3} Nutrient starvation stimulates autophagy via the same canonical pathway. Hence, it is not surprising that also starvation induced changes in the intracellular Ca\textsuperscript{2+} homeostasis and that starvation-induced autophagy was also abolished by BAPTA-AM.

Although these data suggest that Ca\textsuperscript{2+} signaling is an important regulator in the canonical mTOR-controlled autophagy pathway, it remains to be addressed whether other autophagy-inducing stimuli that do not act via this pathway induce similar changes in intracellular Ca\textsuperscript{2+} signaling as rapamycin or
starvation (Fig. 1) or rapamycin treatment. This suggests that (1) Atg5 is not required for enhanced Ca^{2+} signaling during the stimulation of mTOR-controlled autophagy, and (2) the key players responsible for enhanced Ca^{2+} signaling occur upstream of Atg5 in the autophagy pathway. Since LC3 lipidation is downstream of Atg5, it is unlikely LC3 will be a main contributor of the observed changes in Ca^{2+} signaling.

The most intriguing autophagy player in this story however is Beclin 1. We observed that Beclin 1 was essential for sensitization of the Ins(1,4,5)P_3R in HeLa cells after nutrient starvation, resulting in more Ca^{2+} release upon stimulation by low or medium [Ins(1,4,5)P_3]. This sensitization was concomitant with an increased binding of Beclin 1 to the Ins(1,4,5)P_3R and the dissociation of Beclin 1 from inhibitory Bcl-2 family proteins. Beclin 1 knockdown prevented Ins(1,4,5)P_R sensitization during nutrient starvation, while recombinantly expressed and purified Beclin 1 was able to sensitize Ins(1,4,5)P_R-mediated Ca^{2+} flux in non-starved cells. Importantly, Beclin 1 knockdown did not alter the increased ER Ca^{2+}-store content upon nutrient starvation. This indicates that two mechanisms can contribute to the enhanced Ins(1,4,5)P_R-mediated Ca^{2+} signaling during nutrient starvation: the Beclin 1-dependent Ins(1,4,5)P_R sensitization and the Beclin 1-independent increase in ER Ca^{2+} content (Fig. 2). Likely, the more subtle Beclin 1-dependent sensitization of the Ins(1,4,5)P_R is required to generate the more specific autophagy-stimulating Ca^{2+} signals. In this sense, this sensitization may be of more importance than the observed increase in ER Ca^{2+} content, and Beclin 1 may represent the switch that drives the Ca^{2+}-dependent autophagy pathway. Therefore, the role of Beclin 1 in autophagy triggered by different stimuli ought to be further scrutinized.

To have a Look Downstream: The Ca^{2+} Target

An important point that needs clarification is the exact target of these autophagy-stimulating Ca^{2+} signals. The differences observed in autophagy levels after BAPTA-AM or Xestospongin B (an Ins(1,4,5)P_R inhibitor) treatment can partially address this question. BAPTA-AM, which buffers intracellular Ca^{2+}, did not induce any changes in basal autophagy levels, while
Xestospongin B increased them. However, both of them blunted starvation-stimulated autophagy. We anticipate that the different effects on basal autophagy levels by BAPTA-AM and Xestospongin B can be explained by differences in localization of the autophagy-inhibiting and autophagy-stimulating Ca\(^{2+}\) signals (Fig. 2). Ins(1,4,5)\(P_3\)Rs mediate a constitutive Ca\(^{2+}\) release toward mitochondria for assuring an appropriate energy production, which represses AMPK activity and hence keeps autophagy at low levels.\(^5\)

For this pathway, close ER-mitochondria contact sites are required to facilitate the efficient transfer of Ca\(^{2+}\) into the mitochondria.\(^6\) Xestospongin B, but not BAPTA, likely can access these microdomains and inhibit the Ins(1,4,5)\(P_3\)Rs present in the ER-mitochondria contact sites. Hence, only Xestospongin B will attenuate energy production and increase basal autophagy levels. However, since both BAPTA-AM and Xestospongin B can abolish starvation-induced autophagy, the autophagy-stimulating Ca\(^{2+}\) signal has another target than mitochondria.

Several cytosolic Ca\(^{2+}\) targets that regulate autophagy have already been proposed in the literature: CaMKK\(\beta\), PKC\(\theta\) and ERK.\(^9\) However, it should be noted that these reports are based on autophagy-inducing stimuli that result in a rather general increase in the cytosolic [Ca\(^{2+}\)], rather than an increase in the ER Ca\(^{2+}\) content and/or in Ins(1,4,5)\(P_3\)R-mediated Ca\(^{2+}\) release. In particular, thapsigargin, a potent inhibitor of the ER Ca\(^{2+}\)-pump SERCA, is often applied, but this treatment actually leads to an emptying of the ER Ca\(^{2+}\) content, opposite to the effects observed after rapamycin treatment or starvation (Fig. 2). The activation of autophagy through a general increase in cytosolic [Ca\(^{2+}\)] is therefore not strictly equal to autophagy stimulation via increased Ins(1,4,5)\(P_3\)R-mediated Ca\(^{2+}\) signaling, and both ways of autophagy stimulation may have different targets (Fig. 2).

The Beclin 1-mediated Ins(1,4,5)\(P_3\)R sensitization that we observe during nutrient starvation led to a small, but significant increase of Ins(1,4,5)\(P_3\)-induced Ca\(^{2+}\) release.\(^3\) The question arises whether such a small difference in Ca\(^{2+}\) release can have major cellular effects. However, the small nature of this sensitization suggests that the subsequent effects are restricted to the local environment of the Ins(1,4,5)\(P_3\)R. Interestingly, the ER and in particular special subdomains termed omegasomes are reported as important sites of origin for autophagosomes.\(^10\) At these sites several key players for autophagosome formation are assembled, including WIPI proteins and the PI3K complex III. It is therefore possible that the autophagy-inducing Ca\(^{2+}\) signal does not reach beyond the close proximity of its release site, that the target is therefore situated at these omegasomes and that these Ca\(^{2+}\) signals are hence required for the initial steps in autophagosome formation. In this context, a likely Ca\(^{2+}\)-target candidate is CaMKI, involved in the Ca\(^{2+}\)-dependent formation of WIPI-1 punctae upon various triggers of autophagy, including nutrient starvation (Fig. 2).\(^11\)

Recently, more insights in the role of lysosomal Ca\(^{2+}\) homeostasis in autophagy regulation have been obtained. Lysosome-resident NAADP-gated Ca\(^{2+}\) release channels seem important regulators of autophagy.\(^12,13\) Since lysosomes function only in the final steps of the autophagy process, it is likely that Ca\(^{2+}\) release from these organelles predominantly regulates distal steps in the autophagy process, such as autophagosome-lysosome fusions, the degradation of the autophagic cargo or the recycling of the lysosomal membranes. However, Ca\(^{2+}\) release from the lysosomes have been proposed to be amplified by Ca\(^{2+}\) release from the ER by a process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release, and may therefore also modulate proximal steps in the autophagy pathway.\(^14,15\) Moreover, recent work of different groups highlighted the possibility of complex, bi-directional functional interactions between lysosomal and ER Ca\(^{2+}\).\(^16-18\) Thus, enhanced Ins(1,4,5)\(P_3\)R-mediated Ca\(^{2+}\) signaling may not only affect early steps in the autophagy pathway by regulating systems locally at the ER, but also distal steps by reciprocal Ca\(^{2+}\) signaling between the ER and the lysosomes. Thus, the modulation of intracellular Ca\(^{2+}\) signaling at the spatiotemporal level allows for a complex and fine-tuned control of the autophagy pathway by Ca\(^{2+}\), likely involving multiple Ca\(^{2+}\) targets at different steps of the autophagy process.

In conclusion, the present findings represent only the tip of the iceberg in unraveling the role of Ca\(^{2+}\) in autophagy stimulation. The identification of the exact key players, both upstream and downstream of the autophagy-stimulating Ca\(^{2+}\) signals, will
be a key future prospect to further understand the generality and importance of intracellular Ca\textsuperscript{2+} signaling in autophagy regulation. Moreover, the clarification of this complex regulation may provide novel opportunities to modulate autophagy.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.