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Intracellular Ca^{2+} signaling

A novel player in the canonical mTOR-controlled autophagy pathway

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Abbreviations: AMPK, AMP-activated kinase; Atg, Autophagy protein; ATP, adenosine triphosphate; BAPTA(-AM), 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (-acetoxymethyl ester); Bcl-2, B cell CLL/lymphoma 2; CaMKI, Calmodulin kinase I; CaMKKβ, Calmodulin kinase 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_3R, 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; PI3K, Phosphatidylinositol 3-kinase; PKCθ, Protein kinase C θ; SERCA, Sarco-/endoplasmic reticulum Ca^{2+} ATPase; ULK1, Unc-51-like kinase 1; WIPI-1, WD repeat domain phosphoinositide-interacting protein 1

Functional intracellular Ca^{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^{2+}-signaling machinery coincide with autophagy stimulation. This results in enhanced intracellular Ca^{2+} signaling essential for driving the autophagy process. Yet, the mechanisms upstream (the players causing the changes in Ca^{2+} signaling) and downstream (the targets of the altered Ca^{2+} signals) of this Ca^{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^{2+} Signaling

The role of Ca^{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^{2+} on autophagy.[1] We recently investigated the role of intracellular Ca^{2+} homeostasis/signaling in canonical mTOR-controlled autophagy upon rapamycin treatment.[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. Rapamcyin 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^{2+} content and increased Ins(1,4,5)P_3R-mediated Ca^{2+} signaling. Buffering the intracellular Ca^{2+} with the cell-permeable chelator BAPTA-AM abolished rapamycin-induced autophagy, indicating that intracellular Ca^{2+} signaling is required for activation of the canonical autophagy pathway. This study followed up on a previous study investigating the role of Ca^{2+} signaling in nutrient starvation-induced autophagy.[3] Nutrient starvation stimulates autophagy via the same canonical pathway. Hence, it is not surprising that also starvation induced changes in the intracellular Ca^{2+} homeostasis and that starvation-induced autophagy was also abolished by BAPTA-AM.

Although these data suggest that Ca^{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^{2+} signaling as rapamycin or

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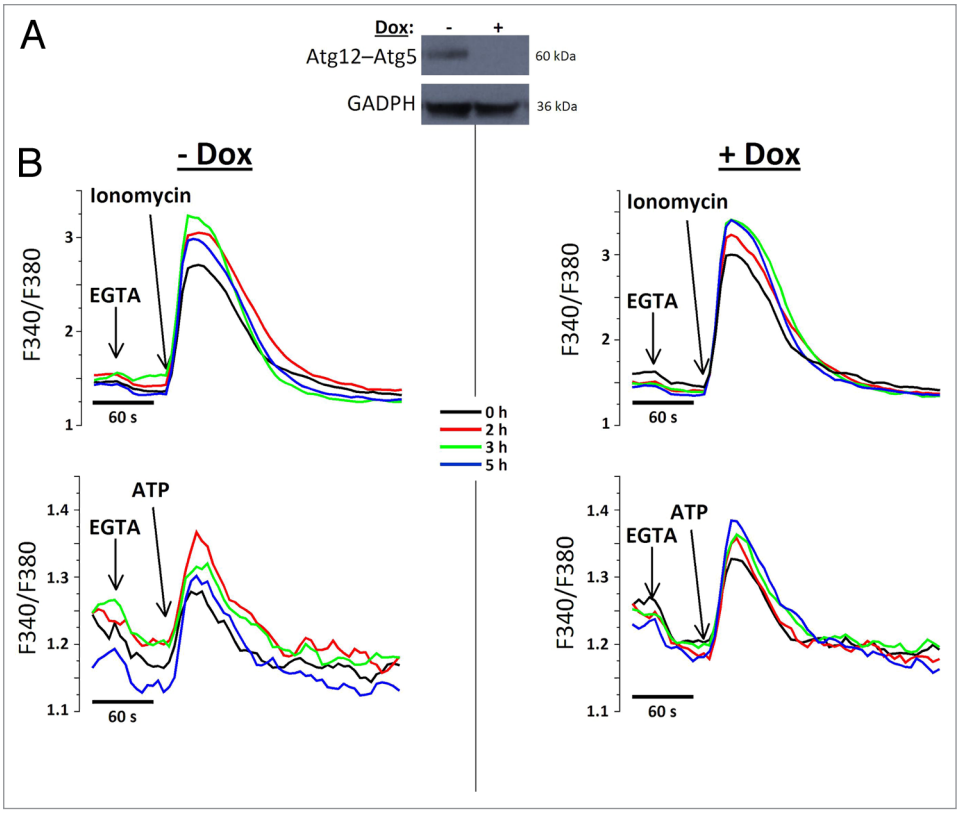

Figure 1. Nutrient starvation-induced changes in Ca^{2+} homeostasis and signaling still occur in Atg5 knockout MEF cells. (**A**) The addition of doxycycline (Dox) (10 ng/ml for 48 h) results in the suppression of Atg5 expression, as shown by the western blot of GADPH and the Atg12–Atg5 protein complex. (**B**) After treatment with doxycycline, cells were starved for the indicated time with Hanks' Balanced Salt Solution and subsequently loaded with the fluorescent Ca^{2+} indicator Fura2-AM. The excitation ratio between 340 and 380 nm (F340/F380) determines the amount of Ca^{2+} in the cytosol. First, 3 mM EGTA was given to chelate the extracellular Ca^{2+}, 45 s later, 10 μM ionomycin (upper row) or 30 μM ATP (lower row) was added to the medium to assess the intracellular Ca^{2+}-store content and the Ins(1,4,5)P_3-induced Ca^{2+} release, respectively. Starved cells demonstrated an increased Ca^{2+} release induced by either ionomycin or ATP. The traces shown are representative examples of n = 5 (ionomycin) or n = 3 (ATP) independent experiments.

starvation. This is certainly not the case for Li^+, which activates autophagy in an mTOR-independent fashion, through inhibition of the inositol metabolism.[4] This leads to less Ins(1,4,5)P_3 and less activation of the ER-resident Ca^{2+} channel Ins(1,4,5)P_3R. Hence, Li^+ works in an opposite way than rapamycin or starvation, i.e., through a decrease in intracellular Ca^{2+} signaling. This discrepancy can be explained by a difference in destination of the Ca^{2+} signals (mitochondria vs. cytosol, see further).[5] This effect of Li^+ clearly exemplifies that autophagy stimulation in general does not always require increased Ca^{2+} signaling. Moreover, when Ca^{2+} signaling participates in the autophagy process, its action will depend on the exact stimulus and on the different autophagy players involved.

To address which of these players are crucial for Ca^{2+}-dependent autophagy stimulation, it will be of interest to evaluate the Ca^{2+} effects in a context of mTOR-, ULK1-, Beclin 1-, Atg5- or Atg7-independent autophagy stimulation. In this respect, it is important to note that the changes in Ca^{2+} homeostasis were still observed in an Atg5-knockout model of MEF cells upon nutrient starvation (**Fig. 1**) or rapamycin treatment.[2] 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].[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.[3] Beclin 1 knockdown prevented Ins(1,4,5)P_3R sensitization during nutrient starvation, while recombinantly expressed and purified Beclin 1 was able to sensitize Ins(1,4,5)P_3R-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_3R-mediated Ca^{2+} signaling during nutrient starvation: the Beclin 1-dependent Ins(1,4,5)P_3R 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_3R 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_3R inhibitor) treatment can partially address this question.[3] 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_3Rs 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_3Rs 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,[3] the autophagy-stimulating Ca^{2+} signal has another target than mitochondria.

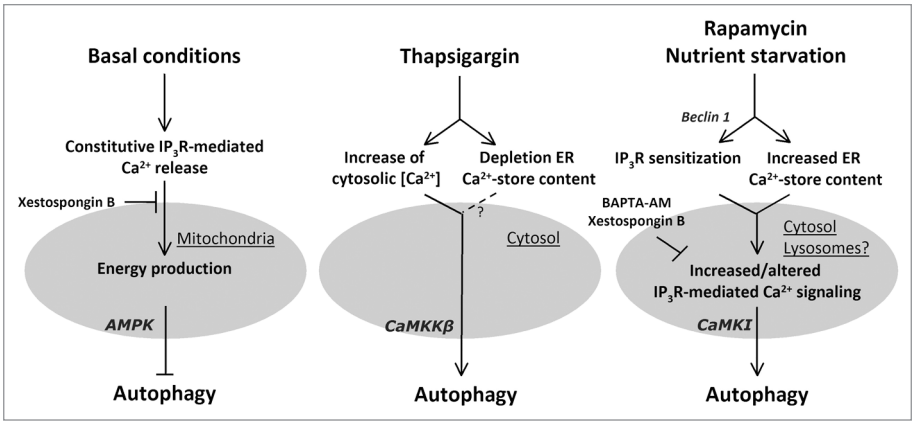

Figure 2. The different mechanisms by which autophagy is controlled by intracellular Ca^{2+}-signaling events. (Left) In basal conditions, constitutive Ins(1,4,5)P_3R-mediated Ca^{2+} release toward mitochondria enhances mitochondrial ATP production, inhibiting autophagy through inactivation of AMPK. This Ca^{2+} signal can be abrogated by Xestospongin B, but not by BAPTA. (Middle) Upon addition of Ca^{2+}-mobilizing agents, like thapsigargin, the ER Ca^{2+} store is emptied and the cytosolic [Ca^{2+}] gradually increases. The latter activates autophagy through a pathway putatively involving CaMKKβ and AMPK. Right: Upon rapamycin treatment or after nutrient starvation, the Ins(1,4,5)P_3R becomes sensitized through the recruitment of Beclin 1 and the increased ER Ca^{2+}-store content, both enhancing Ins(1,4,5)P_3R-mediated Ca^{2+} signaling, thereby stimulating autophagy. These Ca^{2+} signals are not only suppressed by Xestospongin B but also by BAPTA, implying these occur outside the ER-mitochondria microdomains. The target may hence be cytosolic and localized at the ER (e.g., CaMKI) or may be connected with the lysosomes.

Several cytosolic Ca^{2+} targets that regulate autophagy have already been proposed in the literature: CaMKKß,[7] PKCθ[8] 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_3R-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_3R-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_3R 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_3R. 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_3R-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^{2+} signaling in autophagy regulation. Moreover, the clarification of this complex regulation may provide novel opportunities to modulate autophagy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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