# The Inositol Trisphosphate Receptor in the Control of Autophagy

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Addendum to:

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## ABSTRACT

The second messenger *myo*-inositol-1,4,5-trisphosphate ($IP_3$) acts on the $IP_3$ receptor ($IP_3R$), an $IP_3$-activated $Ca^{2+}$ channel of the endoplasmic reticulum (ER). The $IP_3R$ agonist $IP_3$ inhibits starvation-induced autophagy. The $IP_3R$ antagonist xestospongin B induces autophagy in human cells through a pathway that requires the obligate contribution of Beclin-1, Atg5, Atg10, Atg12 and hVps34, yet is inhibited by ER-targeted Bcl-2 or Bcl-$X_L$, two proteins that physically interact with $IP_3R$. Autophagy can also be induced by depletion of the $IP_3R$ by small interfering RNAs. Autophagy induction by $IP_3R$ blockade cannot be explained by changes in steady state levels of $Ca^{2+}$ in the endoplasmic reticulum (ER) and the cytosol. Autophagy induction by $IP_3R$ blockade is effective in cells lacking the obligate mediator of ER stress IRE1. In contrast, IRE1 is required for autophagy induced by ER stress-inducing agents such a tunicamycin or thapsigargin. These findings suggest that there are several distinct pathways through which autophagy can be initiated at the level of the ER.

## INTRODUCTION

Autophagy is now increasingly viewed as a homeostatic mechanism that allows cells to cope with nutrient stress and/or to degrade superfluous material and damaged organelles, hence avoiding cell death.[1-3]

One particularly efficient stimulus of autophagy is stress affecting the endoplasmic reticulum (ER).[4] ER stress is usually caused by the accumulation of incorrectly folded proteins in the ER lumen, giving rise to the so-called unfolded protein response (UPR). ER stress can be induced by overexpressing proteins that contain polyglutamine tracts or by the additions of drugs such as tunicamycin (an inhibitor of N-glycosylation) or thapsigargin (an inhibitor of the ER $Ca^{2+}$ ATPase). Recent work has clearly demonstrated that ER stress causes autophagy throughout evolution, meaning that yeast (*Saccharomyces cerevisiae*) activate autophagy upon stimulation of ER stress with dithiothreitol or tunicamycin.[5] Knock-out of several of the essential autophagy (*ATG*) genes *ATG1*, *8*, *9*, *16* or *20*, abolishes the survival of yeast cells exposed to tunicamycin,[6] indicating that autophagy is indeed required to survive severe ER stress throughout evolution.

Similarly, in mammalian cells, ER stressors such as thapsigargin or tunicamycin are particularly efficient in killing cells when autophagy is inhibited by addition of 3-methyladenine or by removal of the *atg5* gene.[7] ER stress induced by expanded polyglutamine 72 repeat (polyQ72) aggregates is particularly lethal in cells in which the induction of autophagy is curtailed due to the absence of ATG5.[8] In mammalian cells, it was found that the combination of ER stress and autophagy inhibition triggers biochemical hallmarks of apoptosis such as caspase activation.[7,8]

The three major transducers of the UPR are IRE1, PERK and ATF6, which all sense the presence of unfolded proteins in the ER lumen and transduce signals to the nucleus or cytosol. In one study, it was found that mouse embryonic fibroblasts (MEF) that lack IRE1$\alpha$ (but not MEF lacking PERK or ATF6) were unable to mount an autophagic response to ER stress.[7] In contrast, another study reported that ER stress induced by polyQ72 caused autophagy in a fashion that was inhibited by dominant-negative PERK, as well as replacement of the PERK substrate eIF2$\alpha$ by a nonphosphorylable mutant (eIF2$\alpha^{S51A/S51A}$).[8] Irrespective of these details, it appears clear that ER stress is one major stimulator of an autophagic response that participates in the degradation of unfolded proteins and in the removal of superfluous ER membranes.

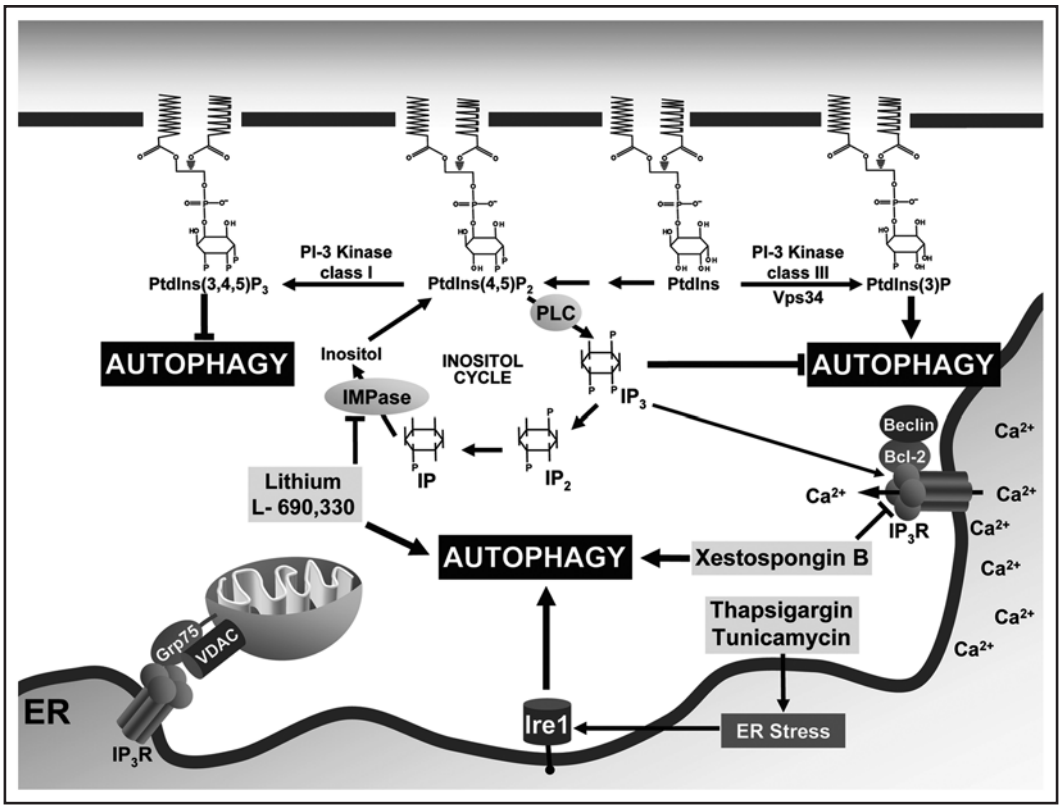


Figure 1. Regulation of autophagy by inositol derivatives. The upper part of the scheme illustrates the metabolism of the most prominent phosphatidyl inositol (PtdIns) derivatives, which can be autophagy inhibitory (in the case of PtdIns(3,4,5)$P_3$) or required for autophagy (such as PtdIns(3)P generated by the class III PI-3 kinase Vps34). Phopholipase C can liberate soluble $IP_3$ from membrane-bound PtdIns(4,5)$P_2$. $IP_3$ is metabolized through dephosphorylation reactions in the inositol cycle. $IP_3$ acts on the $IP_3R$ in the ER membrane. $IP_3R$ acts normally as an endogenous inhibitor of autophagy. Its depletion or inhibition by xestospongin B is likely to affect its interaction with Bcl-2, which in turn inhibits Beclin-1. IRE1α is required for autophagy induction by ER stressors such as thapsigargin or tunicamycin, yet dispensable for autophagy induction by $IP_3R$ inhibition. It is not clear why $IP_3R$ inhibition can induce autophagy of both ER and mitochondria. However, $IP_3R$ is part of a multiprotein complex formed in contact sites between mitochondria and the ER.

Recently, we discovered a different pathway that stimulates autophagy at the level of the ER and that involves the $IP_3R$.[9] It has been demonstrated that inositol derivatives play a major role in the control of autophagy (Fig. 1). One of the endogenous inhibitors of autophagy is *myo*-inositol-1,4,5-trisphosphate ($IP_3$), a second messenger produced primarily in response to the stimulation of G-protein-coupled receptors or receptor tyrosine kinases. Cell-permeable $IP_3$ inhibits the induction of autophagy by agents that inhibit inositol monosphosphatase (such as lithium and L-690,330).[10,11] Cell-permeable $IP_3$ is also a potent inhibitor of autophagy induced by nutrient depletion,[9] yet has no effect on rapamycin-induced autophagy.[10] $IP_3$ acts on the $IP_3R$, a mostly ER-sessile $Ca^{2+}$ release channel that integrates signals from numerous small molecules and proteins including nucleotides, kinases, phosphatases, as well as nonenzyme proteins.[12] $IP_3R$ plays a major role within the $Ca^{2+}$ microdomains that transmit $Ca^{2+}$ spikes generated by the ER to mitochondria,[13] and $IP_3R$ is regulated by Bcl-2 and Bcl-$X_L$, which affect $Ca^{2+}$ fluxes through $IP_3R$ by direct molecular interactions, by influencing its regulatory phosphorylation and/or by modulating its response to $IP_3$. Through these mechanisms, $IP_3R$ modulates a diverse range of cellular functions, which include, but are not limited to, gene expression, contraction/excitation, cellular growth and apoptosis.

Inhibition of $IP_3R$ with a specific antagonist, xestospongin B, or knockdown of different $IP_3R$ isoforms with small interfering RNAs are strong stimuli of autophagy.[9] The autophagic pathway triggered by $IP_3R$ inhibition follows a canonical pathway thus far in that it requires the obligate contribution of Beclin-1, Atg5, Atg10, Atg12 and hVps34. $IP_3R$ inhibition does not elicit ER stress. While typical ER stressors (such as thapsigargin, or tunicamycin) increase the expression of the ER stress indicator GADD34, $IP_3R$ inhibition by xestospongin B failed to induce GADD34.[9] Moreover, xestospongin B induces autophagy (measured as aggregation of LC3-GFP in cytosolic vacuoles) in IRE1α knockout MEFs to a similar degree as in wild type MEFs (Fig. 2A). These results were further confirmed by the siRNA-mediated specific knockdown of IRE1α in HeLa cells (Fig. 2C). Thus, at difference with thapsigargin or tunicamycin, which do require IRE1α for the stimulation of autophagy, $IP_3R$ inhibition by xestospongin-B can trigger autophagy in the absence of IRE1α (Fig. 2A). The inhibition of autophagy by ER stress inducers correlates with a greater loss of $\Delta\Psi_m$ and viability in IRE1α KO MEFs than in WT MEFs. $IP_3R$ inhibition in contrast, does not significantly increase cell death in IRE KO MEFs compared to WT MEFs (Fig. 2B). A further difference between autophagy induced by ER stressors and autophagy induced by $IP_3R$ inhibition resides in the organelles that are secluded by phagophores. ER stress causes preferential autophagy of the ER, meaning that autophagic vacuoles mostly colocalize with the ER marker calreticulin,[14] but not with the mitochondrial matrix marker HSP60. In contrast, $IP_3R$ inhibition causes autophagy of both ER and mitochondria.[9]

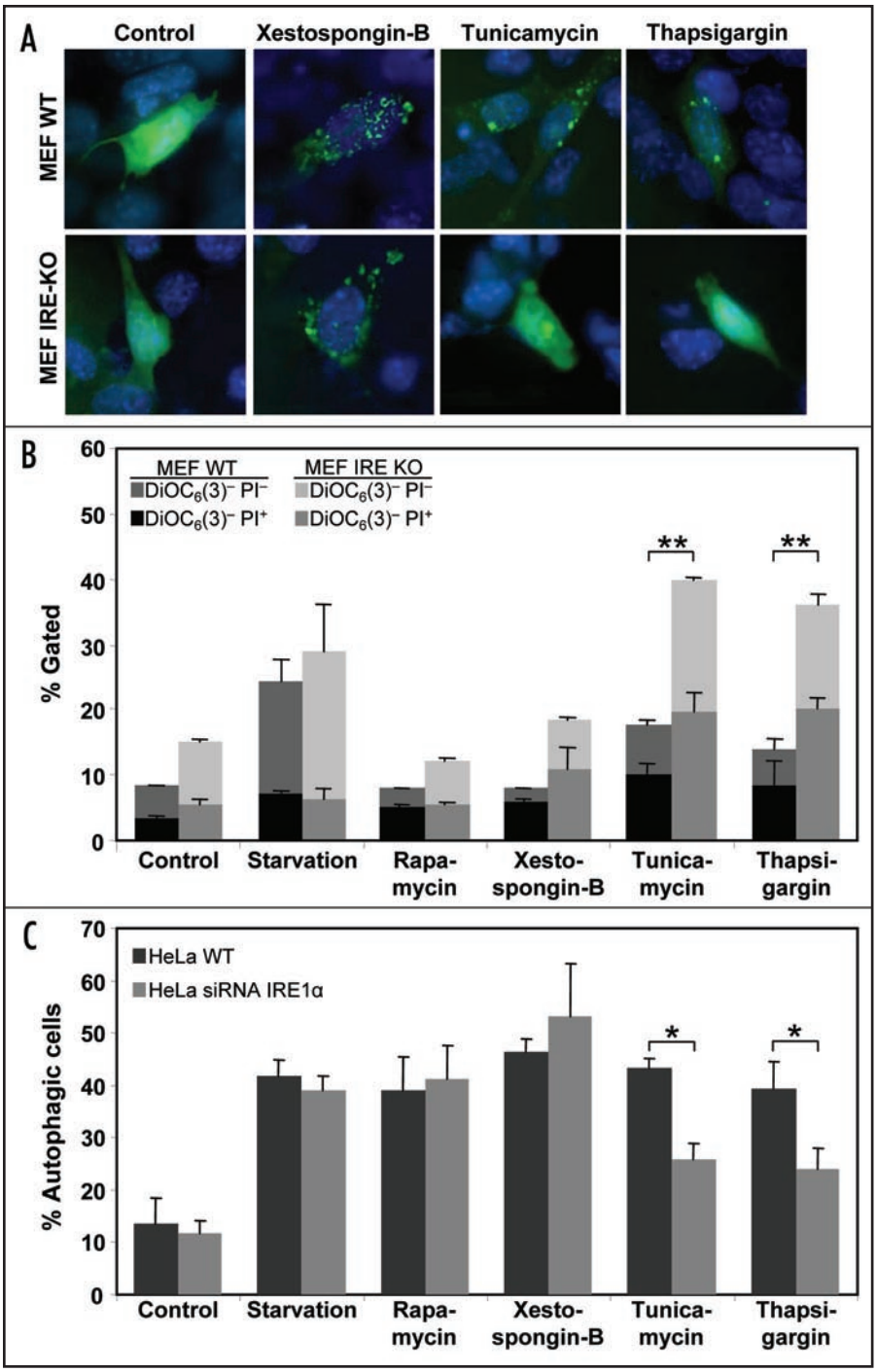


Figure 2. Differential effects of IRE1α on the autophagic response induced by ER stressors and $IP_3R$ inhibition. WT MEFs, IRE1α-deficient MEFs, HeLa cells or siRNA-mediated IRE1α-knock down HeLa cells were transfected with an LC3-GFP reporter construct. Thirty-six hours latter, the cells were cultured for 12 hours in the absence or presence of rapamycin (10 μM), xestospongin B (2 μM), thapsigargin (3 μM) or tunicamycin (2.5 μM). (A) Fluorescence microscopy images of WT or IRE1α knockout (KO) MEFs showing LC3-GFP distribution. The blue fluorescence corresponds to Hoechst 33342 [10 μg/ml] stained nuclei. (B) The frequency of adherent and nonadherent cells with a low $\Delta\Psi_m$ was determined by cytofluorometry after staining with 3,30-dihexyloxacarbocyanine iodide ($DiOC_6(3)$ [40 nM]) and counterstaining with the vital dye propidium iodide (PI [1 μg/ml]). Asterisks indicate significant differences between $DiOC_6(3)^-$ $IP^-$ groups as indicated (**p < 0.01). (C) The percentage of HeLa cells exhibiting redistribution of LC3-GFP to cytosolic vacuoles was scored as an indication of autophagy. A mixture of two specific siRNAs targeted to *IRE1α* (sense 5'-GCCCGGCCUCGGGAUUUUU-3' and sense 5'-GCGUCUUUUACUACGUAAU-3') was used to deplete IRE1α by transfection with Oligofectamine 48 h before the addition of the indicated agents. Asterisks indicate significant differences (*p < 0.05). All results are representative of two independent experiments. Means ± standard deviations of triplicates are shown.

ER stress and $IP_3R$ inhibition differs also with respect to its regulation by Bcl-2 and Bcl-$X_L$. Transfection-enforced overexpression of Bcl-2 or Bcl-$X_L$ fails to prevent autophagy induced by thapsigargin or tunicamycin, yet efficiently suppresses autophagy induced by xestospongin B.[9] This inhibitory effect is particularly strong when Bcl-2 or Bcl-$X_L$ are targeted to the ER, yet are lost when these proteins are targeted to mitochondria. At present, the precise mechanisms through which Bcl-2 or Bcl-$X_L$ inhibit autophagy at the ER level are elusive. According to one report[15] Bcl-2 or Bcl-$X_L$ reduce the lumenal ER $Ca^{2+}$ concentration, which in turn blunts cytosolic $Ca^{2+}$ fluxes induced by agonists such as thapsigargin. This would then interrupt a signal transduction pathway in which an increase in cytosolic $Ca^{2+}$ sequentially activates the $Ca^{2+}$/calmodulin-dependent kinase kinase-β (CAMKK-β) and AMP-activated protein kinase (AMPK), which then would inhibit mammalian target of rapamycin (mTOR) and hence stimulate autophagy.[15] However, this pathway probably does not apply to the Bcl-2/Bcl-$X_L$-mediated modulation of xestospongin B-induced autophagy, because xestospongin B fails to affect the cytosolic and lumenal ER $Ca^{2+}$ concentration in conditions in which it is highly effective in inducing autophagy.[9] Based on our data, we prefer another scenario. It has been shown that ER-localized Bcl-2 and Bcl-$X_L$ can regulate autophagy through a direct physical inhibiton of the essential autophagy protein Beclin-1.[16] Upon addition of xestospongin B, the amount of $IP_3R$ that immunoprecipitated with ER Bcl-2 is reduced,[16] indicating that changes in the stoichiometry of autophagy-inhibitory protein complexes might account for the induction (or de-inhibition) of the autophagic process. This possibility is currently under intense investigation in our laboratory.

In summary, $IP_3$ and $IP_3R$ emerge as novel endogenous regulators of autophagy. The challenge is now to understand the mechanistic details through which $IP_3R$ inhibits or unleashes the autophagic machinery and to integrate the couple formed by $IP_3$ and $IP_3R$ in the regulatory circuits that control autophagy in health and disease.

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