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Resveratrol as an inductor of autophagy: is there a unique pathway of activation?

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Autophagy is a process of cellular degradation for the removal of damaged components and eventual recycling of the resulting molecules that help to the maintenance of cellular homeostasis. Through this pathway, proteins with relatively long half-life and whole organelles (e.g., mitochondria, peroxisomes, and ribosomes) are degraded in the cytoplasm for the continuous turnover of cellular structures to facilitate their renewal. However, the beneficial effect of autophagy goes beyond cellular "cleaning" and resides to a large extent on its ability to recycle cell components back into the cytosol. Thus, autophagy is a fundamental mechanism to regulate cellular functions, including DNA repair, proliferation, differentiation, embryonic development, and the immune response.

Autophagy in the nervous system (NS): It is known that autophagy in the NS participates in neuronal differentiation and in the removal of apoptotic cells during the normal NS development, and that allows axonogenesis and neuritogenesis during stem cell differentiation. Recently, autophagy was described as a novel therapeutic option for improving motor and optic nerve regeneration leading to functional recovery after injury. Indeed, autophagy malfunction leads to the accumulation of altered proteins and organelles that interfere with normal cell functioning and has been linked to pathophysiological conditions such as neurodegenerative diseases, metabolic disorders, and cancer. For instance, loss of *Atg7*, a gene essential for autophagy, leads to neurodegeneration and that conducts to abnormal limb-clasping reflexes, reduced coordination of movement, and premature death. Autophagy deficiency also causes a massive neuronal loss in the cerebral and cerebellar cortices and limit the neural regeneration and functional recovery after spinal cord injury.

Resveratrol (3,5,4'-trihydroxystilbene, RSV) as inductor of autophagy: During nutrient starvation, stress or conditions of high metabolic demand, cells activate mechanisms aimed to sense the energetic deficiency and provide macromolecules for survival. Interestingly, the use of drugs or compounds derived from some plants maximizes the translation of information to the intracellular machinery responsible for energy generation through autophagy. Among these compounds, RSV a natural polyphenolic present in grapes, blueberries, and peanuts, is distinguished because it regulates several cellular processes that

prevent cellular injury and increase viability. Importantly, autophagy regulation is responsible for some of the beneficial effects of RSV in different pathologies (Kulkarni and Cantó, 2015; Pineda-Ramirez et al., 2018). Therefore, there is a growing interest in studying the mechanisms of action of RSV to develop a possible therapeutic strategy. To this end, it is important to identify the signaling pathways and targets involved in the autophagy process. Unfortunately, few experimental studies have been carried out to evaluate the route of activation that RSV turns on.

RSV can activate multiple cell-signaling pathways depending on at least four factors: the cell type, the dose used, the stimulation time, and the pathology involved. Autophagy activation by RSV has been demonstrated in different *in vivo* and in vitro pathological models: several cancer types, hepatic steatosis, dietary-induced hepatic lipid accumulation, cardiac hypoxia/reoxygenation, cardiac ischemia/reperfusion, stroke, spinal cord injury, peripheral nerve injury, sciatic nerve crush injury, and neurodegenerative diseases (Huang et al., 2016; Pineda-Ramírez et al., 2018). In these models, RSV modulates metabolic and mitochondrial function, promotes neural regeneration, and restores neural function through specific signaling pathways, although the precise mechanisms involved have not been elucidated yet. Studies in vitro have given very precise information. RSV-induced signaling pathway is not conclusively defined and there is a significant divergence on results obtained among the different laboratories studying its effects. Besides, sometimes is easier to opt for a single route instead to identify the crosstalk signal that RSV activates. Nevertheless, there are distinctive pathways activated by RSV, which strengthens the importance of autophagy in controlling pathogenesis and promoting neural regeneration.

The basic autophagy machinery: The canonic pathway of autophagy regulation consists of inhibition of the Serine/Threonine kinase called the mammalian target of rapamycin (mTOR), a master sensor of nutrient levels in the cell (**Figure 1**). Ordinarily, mTOR interacts with other proteins to form mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is the most studied and its best-characterized targets are the eukaryotic initiation factor 4E binding protein 1 and the 40S ribosomal S6 kinase 1. mTORC1 function depends on the activity of tuberous sclerosis complex protein 1 (TSC1) and 2 (TSC2). Rag

GTPases, like, RagA/B and RagC/D, regulated by the availability of intracellular nutrients, energy, oxygen, and growth factors, also promote the activity of mTORC1 (**Figure 1**). mTORC1 promotes gene translation through the phosphorylation of targets that are involved in proliferation, cellular growth, and survival.

Stimulation of autophagy begins with the inhibition of mTOR that modulates the activity of the complex Atg101/ULK1/FIP200/Atg13, specifically through the inhibitory phosphorylation of ULK1 necessary to initiate the formation of the phagophore. Downstream to this, the phosphatidylinositol-3 kinase class III protein complex (PI3K Complex III), which consists mainly of PI3K, Vps15, Beclin 1 and Atg14 (with different roles depending on whether they are bound to Ambra1, Bif-1/UVRAG, or UVRAG/Rubicon), is dynamically regulated by phosphorylation, and protein-protein interactions that induce or inhibit autophagy. Then, the elongation of the phagophore requires the formation of the Atg5/Atg12/Atg16L1 complex through ubiquitination and the lipidation of the microtubule-associated protein 1 light chain 3 (LC3). LC3-I becomes conjugated to phosphatidylethanolamine to form LC3-II. This lipid tail enables LC3-II insertion into the membrane of the formation of autophagosomes. Finally, the autophagosomes are transported along microtubules in a dynein-dependent manner to the lysosomes. The final fusion of autophagosomes with lysosomes is regulated by the endosomal complex required for transport III, SNAREs, Rab7, and class C Vps proteins (**Figure 1**; Mizushima and Komatsu, 2011).

Pathways activated by RSV to induce autophagy: In several studies, the protective functions of RSV have been associated with regulation of metabolic sensors/effectors including mTOR, the adenosine-5-monophosphate-activated protein kinase (AMPK), Sirtuin 1 (SIRT1), the poly-ADP-ribose polymerase 1, the PI3K/protein kinase B pathway (Akt), as well as the mitogen associated protein kinase (MAPK) signaling pathways. All of these players are essential in the process that allows autophagy. Nonetheless, as in many other processes in which RSV participates, the exact mechanism has not been described.

mTOR is a direct target of RSV. mTOR activity is inhibited in an ATP-competitive and a dose-dependent manner preventing the phosphorylation of mTOR' substrates. Additionally, low concentrations of RSV lead to phosphorylation of mTOR at serine 2481, a residue associated with the increase mTORC2 activity, while high concentrations block mTORC2 phosphorylation, indicating that RSV might have multiple effects in the mTORC2 signaling pathway. RSV also promotes the association between mTOR and DEPTOR (Kulkarni and Cantó, 2015). DEPTOR is a negative regulator of mTOR complexes that inhibits mTOR kinase activity, representing a potential mechanism of
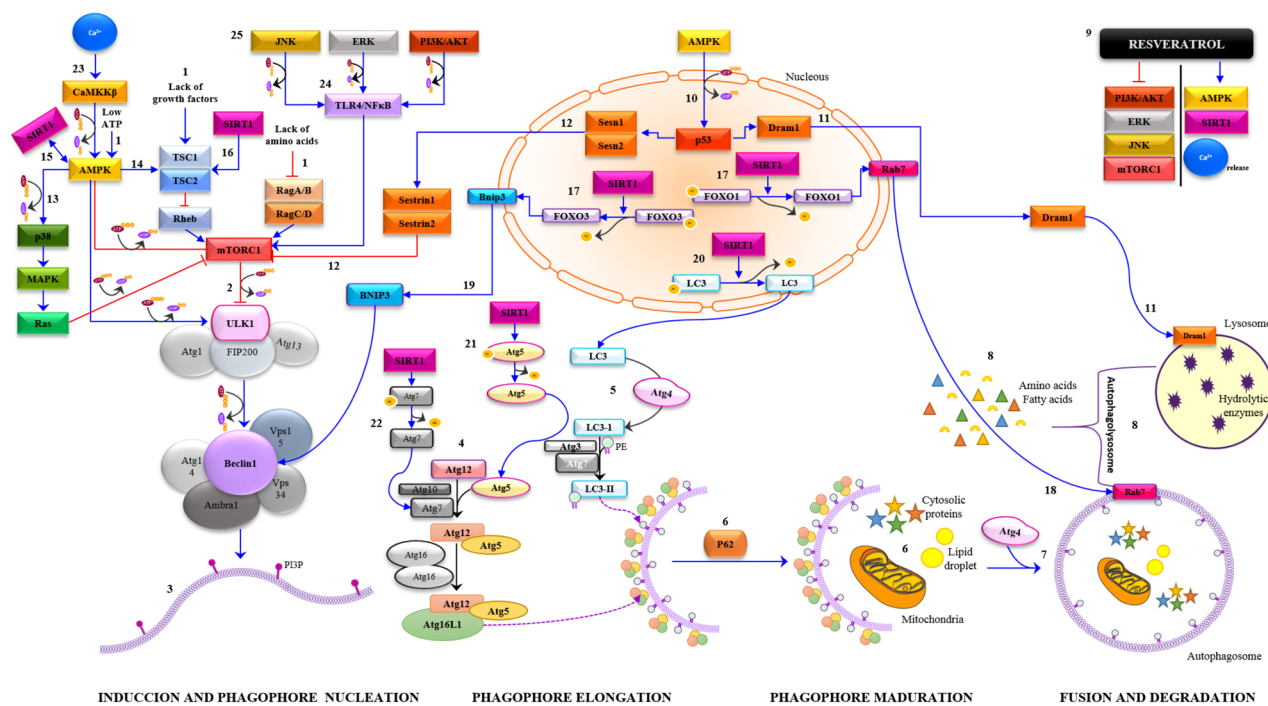
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Figure 1 | Pathways of autophagy regulation.
Activation of autophagy involves the inhibition of the mTORC1 by deprivation of growth-promoting stimuli (growth factors, amino acids) or by AMPK (1). The ULK1 complex regulates the class III phosphatidylinositol (PtdIns) 3-kinase complex (2). Both complexes induce the formation of the phagophore (a double membrane rich in PI3P), which elongates into a vesicle (autophagosome) (3). This elongation requires the Atg12–Atg5–Atg16 complex (4) and lipidation of LC3 (LC3-II), which are inserted in the membrane of the phagophore (5). p62 recruits' proteins, fatty droplets, and/or organelles into the autophagosome (6). Atg4 slides the LC3-II molecules that are located on the outside of the autophagosome membrane and transforming them back into their cytosolic form (7). The autophagosome fuses with the lysosome, forming an autophagolysosome (8). RSV modulates autophagy through AMPK/mTORC1 dependent and independent pathways (9, 10). AMPK phosphorylates nuclear p53, which promotes transcription (11). Dram1 regulates autophagolysosome formation (11). Sestrin1/Sestrin2 and p38MAPK inhibit mTOR (12, 13). TSC1 and TSC2 (mTOR blockers) favor the activation of the SIRT1 pathway (14). SIRT1 activates AMPK by feedback and regulates TSC1 and TSC2 (15, 16). SIRT1, in the nucleus, deacetylates FOXO1 and FOXO3, which promote the transcription of Rab7 (autophagosomes maturation) and Bnip3 (Beclin1 regulator), and deacetylates LC3 (17–20). In the cytoplasm, SIRT1 promotes the deacetylation of the Atg 5 and Atg7, favoring phagophore elongation (21–22). RSV increases autophagy through CaMKKβ (23). Additionally, RSV activates autophagy by decreasing the TLR4/NF-κB pathway that activates mTOR (24), by inhibiting PI3K/Akt, ERK, and JNK (25). Ambra1: Activating molecule in beclin 1-regulated autophagy protein 1; AMPK: adenosine-5-monophosphate-activated protein kinase; Atg: autophagy related; Atg16L1: autophagy related 16 like 1; ATP: adenosine triphosphate; Bnip3: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; Ca^{2+}: calcium ion; CaMKKβ: Ca^{2+}/calmodulin-dependent protein kinase kinase β; Dram1: DNA damage regulated autophagy modulator 1 gene and protein; ERK: extracellular signal-regulated kinase; FIP200: focal adhesion kinase family interacting protein of 200 kD (ULK binding); FOXO1: Forkhead box O1 gene; FOXO3: Forkhead box O3 gene; JNK: c-Jun N-terminal kinase; LC3: microtubule-associated protein 1A/1B-light chain 3; LC3-1: microtubule-associated protein 1A/1B-light chain 3 (without C-terminal); LC3-II: microtubule-associated protein 1A/1B-light chain 3 (lipidated form); MAPK: mitogen-activated protein kinases; mTORC1: mammalian target of rapamycin complex 1; PE: phosphatidylethanolamine; PI3P: phosphatidylinositol-3-phosphate; PI3K/AKT: phosphatidylinositol-3-kinase/protein kinase B; p38: p38 mitogen-activated protein kinases; p53: p53 tumor suppressor gene; P62: sequestosome 1 (SQSTM1); Rab7: Rab7 small GTPase; RagA/B: Rag GTPase heterodimer A/B; RagC/D: Rag GTPase heterodimer C/D; Ras: Ras GTPase; Rheb: Ras homolog enriched in brain; Sesn1: Sestrin gene 1; Sesn2: Sestrin gene 2; SIRT1: Sirtuin 1; TLR4/NFκB: Toll-like receptor 4/nuclear factor kappa B; TSC1: tuberous sclerosis complex protein 1; TSC2: tuberous sclerosis complex protein 2; ULK1: Unc-51 like autophagy activating kinase 1; Vps15: phosphoinositide 3-kinase regulatory subunit; Vps34: vacuolar protein sorting 34.

regulation of mTOR signaling. Importantly, the inhibition of mTOR promotes autophagy activation, axonal regeneration, Schwann cell remyelination, and motor function recovery in models of sciatic nerve crush injury and peripheral nerve injury (Huang et al., 2016).

On the other hand, activation of the mTORC1 can be mediated by the MAPK through Ras-dependent activation of the S6K. In this case, RSV modulates rapamycin-induced autophagy via inhibition of an upstream protein of mTORC1. RSV directly inhibits various enzymes such as PI3K that also regulate mTOR activity (**Figure 1**; Kulkarni and Cantó, 2015). Evidence suggests that RSV acts mainly through the regulation of the activity of the AMPK and PI3K/Akt pathways at different levels. PI3K/Akt pathway regulates cell survival in a wide range of cell types. RSV by inhibiting Akt phosphorylation in a dose- and

time-dependent manner, induces autophagy through regulation of the PI3K/Akt/mTOR pathway. Akt promotes mTORC1 activation by phosphorylation and inhibition of TSC2. In the case of AMPK, this is an important regulator of autophagy. AMPK is a serine/threonine-protein kinase that inhibits mTOR in response to an increase in the AMP/ATP ratio (**Figure 1**). AMPK impacts mitochondrial functions, increasing the NAD$^+$/NADH ratio which in turn, activates SIRT1. SIRT1, a member of the highly conserved family of NAD$^+$-dependent deacetylase that regulates negatively mTORC1, through association with TSC1/TSC2 (**Figure 1**). Also, cytoplasmic Ca^{2+} concentration and Ca^{2+}-handling proteins modulate autophagy and some studies report the activation of AMPK via calmodulin-dependent kinase kinase β (CaMKKβ) (**Figure 1**) (Bootman et al, 2018). RSV regulates

AMPK activity in animal models that reproduce NS damage (e.g., subarachnoid hemorrhage, stroke, and Parkinson's disease), fatty liver disease, and cancer, suggesting that AMPK could be an essential target of RSV to regulate autophagy and prevent damage. In our laboratory, we found that the protective effect of RSV on cerebral ischemia is closely related to the regulation of autophagy through the activation of AMPK (Pineda-Ramírez et al., 2020). However, the participation of this kinase in the induction of survival in ischemia is controversial. This diverse effect might be related to the specific protocol of RSV's administration used and the conditions of the experimental models (Pineda-Ramírez et al., 2018).

In other cases, RSV enhances the autophagy induced by the AMPK/p38MAPK pathway (**Figure 1**), resulting in protection.

Accordingly, activation of p38 is also connected to an increase in autophagic flux (Ulakcsai et al., 2018). RSV inhibits Akt/mTOR pathway while the p38 MAPK pathway is activated in a dose-dependent manner. Therefore, there is increasing evidence to support the effects of RSV on the p38 kinase pathway, although reports are still scarce.

RSV activates autophagy in an mTOR-independent pathway: RSV directly activates SIRT1 and increases its expression, suggesting that RSV can also activate autophagy through an mTOR-independent via. In support of the mTOR-independent mechanism, SIRT1 may induce autophagy directly by deacetylating Atg 5, Atg 7, and LC3 (**Figure 1**) (Huang et al., 2015). Importantly, SIRT1 is an essential target of RSV that regulates autophagy and promotes nerve regeneration in models of sciatic nerve crush injury, traumatic accidents with nerve injury, spinal cord and optic nerve lesions (Ding et al., 2018).

Besides, SIRT1 deacetylates FOXOs and stimulates the expression of autophagy regulatory molecules (**Figure 1**). SIRT1-deacetylated FOXO1 increases the expression of Rab7, a small GTPase that is a crucial factor in the maturation of autophagosomes and endosomes, leading to activation of its transcriptional activity and subsequent Bnip3-mediated autophagy. Furthermore, SIRT1 may indirectly induce autophagy via direct activation of LKB1/AMPK/ULK1 (**Figure 1**). This evidence supports that RSV regulates autophagy through mTOR independent mechanisms.

In our laboratory, we have demonstrated that RSV enhances the expression of autophagy proteins (Becn1 and LC3) through the activation of AMPK (Pineda-Ramirez et al., 2020). Specifically, RSV induces autophagy through the phosphorylation of p53 on Ser15 by AMPK activation (**Figure 1**). Protein p53 can activate or inhibit autophagy depending on its subcellular localization (Suvorova et al., 2018): cytoplasmic p53 inhibits autophagy, while nuclear p53 stimulates autophagy through its target genes (*Dram1*, *Sesn1*, and *Sesn2*). DNA damage-regulated autophagy modulator 1 (*Dram1*) gene encodes a Dram1 lysosomal protein that increases lysosomal acidification, regulates fusion of lysosomes with autophagosomes and favors the clearance of autophagosomes. RSV up-regulates Dram1 gene expression through nuclear p53 indicating its pro-autophagic activity. *Sesn1* and *Sesn2* genes, which encode Sestrin1 and Sestrin 2 proteins, respectively, negatively regulate the mTOR pathway (**Figure 1**). RSV induces a slight increase in *sesn1* and *sesn2* gene expression suggesting that mTOR-dependent regulation of autophagy by p53 is not the primary pathway. This finding, while reinforcing the importance of RSV in regulating autophagy through AMPK and SIRT1 suggest a distinctive mechanism downstream through regulation of p53 activity that provides a balance between cell viability and cell death.

Other studies propose, RSV mediates the activation of neuronal autophagy by inhibition of the Toll-like receptor 4/nuclear factor enhancer of the light chains kappa of activated B cells (NF-κB) pathway (**Figure 1**) (Feng et al., 2016). RSV inhibits Toll-like receptor 4/NF-κB pathway by down-regulating the activity or the expression of other molecules, like the Signal Transducer and Activator of Transcription 3, the Myeloid Differentiation primary response 88 protein, the TNF receptor associated factor 6, the c-Jun N-terminal kinases, the extracellular signal-regulated kinase 1/2, Akt, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha, and NF-κB (**Figure 1**) (Min et al., 2018). However, the role of the Toll-like receptor 4/NF-κB pathway in autophagy activation remains unclear. In some types of cancer, suppression of TNF receptor associated factor 6 expression leads to the activation of autophagy. As discussed above, the current knowledge appears to suggest that RSV acts through multiple pathways making useless trying to define a single mechanism.

Conclusion: RSV induces the autophagy pathway and plays an essential role in the alleviation of induced injury in many experimental models of disease. The benefits of RSV that have been described range from the energetic regulation of the cell (promoting ATP production) to the regulation of neural regeneration, and the recovery of its function. This information may serve as a basis for the development of future therapeutic strategies. However, more investigation is needed to understand whether independent pathways and pathways with crosstalk exist, because RSV mechanisms of action appears particularly complex. Regardless of the signaling mechanism involved, we consider that RSV is highly attractive to explore its therapeutic potential for preventing neuronal injury and other multiple diseases through autophagy induction.

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References

Bootman MD, Chehab T, Bultynck G, Parys JB, Rietdorf K (2018) The regulation of autophagy by calcium signals: Do we have a consensus? Cell Calcium 70:32-46.

Ding Z, Cao J, Shen Y, Zou Y, Yang X, Zhou W, Guo Q, Huang C (2018) Resveratrol promotes nerve regeneration via activation of p300 acetyltransferase-mediated VEGF signaling in a rat model of sciatic nerve crush injury. Front Neurosci 12:341.

Feng Y, Cui Y, Gao J, Li M, Li R, Jiang X, Tian Y, Wang K, Cui C, Cui J (2016) Resveratrol attenuates neuronal autophagy and inflammatory injury by inhibiting the TLR4/NF-κB signaling pathway in experimental traumatic brain injury. Int J Mol Med 31:921-930.

Huang H, Chen L, Zhang H, Li S, Liu P, Zhao T, Li C (2016) Autophagy promotes peripheral nerve regeneration and motor recovery following sciatic nerve crush injury in rats. J Mol Neurosci 58:416-423.

Huang R, Xu Y, Wan W, Shou X, Qian J, You Z, Liu B, Chang C, Zhou T, Lippincott-Schwartz J, Liu W (2015) Deacetylation of nuclear LC3 drives autophagy initiation under starvation. Mol Cell 57:456-466.

Kulkarni SS, Cantó C (2015) The molecular targets of resveratrol. Biophys Acta 1852:1114-1123.

Min Y, Kim M, Lee S, Chun E, Lee K (2018) Inhibition of TRAF6 ubiquitin-ligase activity by PRDX1 leads to inhibition of NFKB activation and autophagy activation. Autophagy 14:1347-1358.

Mizushima N, Komatzu M (2011) Autophagy: renovation of cells and tissues. Cell 147:728-738.

Pineda-Ramírez N, Alquisiras-Burgos I, Ortiz-Plata A, Rauiz-Tachiquín ME, Espinoza-Rojo M, Aguilera P (2020) Resveratrol activates neuronal autophagy through AMPK in the ischemic brain. Mol Neurobiol 57:1055-1069.

Pineda-Ramírez N, Gutiérrez Aguilar GF, Espinoza-Rojo M, Aguilera P (2018) Current evidence for AMPK activation involvement on resveratrol-induced neuroprotection in cerebral ischemia. Nutr Neurosci 10:1-19.

Suvorova II, Knyazeva AR, Pospelov VA (2018) Resveratrol-induced p53 activation is associated with autophagy in mouse embryonic stem cells. Biochem Biophys Res Commun 503:2180-2185.

Ulakcsai Z, Bagaméry F, Szökő É, Tábi T (2018) The role of autophagy induction in the mechanism of cytoprotective effect of Resveratrol. Eur J Pharm Sci 123:135-142.