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REVIEW ARTICLE

Autophagy in hypoxia-ischemia induced brain injury

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Autophagy is an endogenous tightly regulated process responsible for the degradation of damaged and dysfunctional cellular organelles and protein aggregates. Emerging data indicate a strong and complex interaction among autophagy, apoptosis and necrosis. We studied these interactions in a neonatal model of hypoxia-ischemia (HI). Autophagy was assessed by evaluating the expression of the two autophagy proteins beclin 1 and LC3, and by "*in vivo*" autophagic vesicles formation and clearance using monodansylcadaverine (MDC). Both autophagy and apoptosis pathways were increased in the same neurons at short times after HI. Neuroprotective drugs also increased autophagy. Interestingly, pharmacological inhibition of autophagy switched cell death phenotypes from apoptosis to necrosis. Rapamycin, that enhances autophagy by inhibition of mTOR and previously shown to be neuroprotective in our animal model of HI when administered before the ischemic insult, was used to study the potential interaction between autophagy and survival pathways. Rapamycin, besides inducing autophagy, also increased Akt and CREB (cAMP response element-binding protein) phosphorylation in the same cells. The pharmacological inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt axis reduced the neuroprotective effect of rapamycin without affecting autophagy. Conversely, pharmacological inhibition of autophagy reduced the neuroprotective effect of rapamycin without affecting Akt phosphorylation. Both treatments, however, caused a rapid switch towards necrotic cell death. Thus, autophagy can be part of an integrated pro-survival signalling which includes the PI3K-Akt- mTOR axis and its activation seems be crucial for pharmacological and ischemic preconditioning.

Keywords: autophagy, apoptosis, necrosis, hypoxic-ischemic encephalopathy, neuroprotection, neonate, hypoxia, ischemia

Introduction

Autophagy is a self-degradation process that is essential for survival, differentiation, development and homeostasis. There are three different forms of autophagy, chaperone-mediated autophagy, microautophagy, and macroautophagy, that differ in their mechanisms, physiological functions and cargo specificity. Macroautophagy (hereinafter referred to as autophagy), has recently become the best-studied form of autophagy because contributes to maintain the balance between degradation, synthesis, and recycling of cellular components, and is strictly linked to cell death pathways [1–3]. During the autophagy process parts of the cytoplasm, long-lived proteins and intracellular organelles are sequestered within cytoplasmic double-membrane vesicles – the autophagosomes or autophagic vacuoles – and delivered to lysosomes for bulk degradation (Figure 1). Autophagy is induced during different stress responses, including starvation, oxidative stress and hypoxia [4–6] and is associated with the pathological mechanisms involved in many diseases. The presence of autophagosomes in dying cells has implicated autophagy in the cell death process. Indeed, excessive autophagic activity may destroy proportions of the cytosol and organelles, leading to collapse of all cellular functions. However, autophagy also represent an adaptive strategy by which cells clear damaged organelles and survive nutritional bioenergetics stress and its activation is critical during mammalian development when nutrients are restricted. Indeed, immediately after birth autophagy is up-regulated in various tissues returning to basal level within 1–2 days, indicating that it is an important mechanism for survival during neonatal starvation [7]. Herein we review data obtained in our lab that concern the role of autophagy in neonatal hypoxic-ischemic brain injury.

Autophagy is induced after hypoxic-ischemic brain injury

Autophagy is markedly activated after neonatal HI. Beclin 1, a component of the phosphatidylinositol 3-kinase (PI3K) complex that is required for autophagy, and LC3, a microtubule-associated protein that is lipidated upon activation of autophagy, rapidly increase in cells of the injured side [8,9]. To further assess autophagy activation in neonatal HI, we recently set up a new method for measuring autophagy that was based on the *in vivo* administration of monodansylcadaverine (MDC), a compound known to label acidic endosomes, lisosomes and autophagosomes [10,11]. No MDC labeling was observed in control animals or in the unlesioned side of the brain of the ischemic animals, whereas a marked MDC labeling was observed in the lesioned side starting from 2 h from the hypoxic-ischemic insult. MDC labeling co-localized with LC3-II confirming that the autophagy flux is increased in hypoxic-ischemic conditions in the neonate. Autophagy is activated in neurons since beclin-1 shows a strong co-localization with the neuronal-specific marker MAP2 but not with GFAP, a marker of astrocytes, or ED1, a marker for activated microglia/ macrophages. However, autophagy is also activated in glial cells, but at later times [12,13]. The increased activation of autophagy in ischemic conditions, however, is not a peculiar feature of the neonate and is also observed in different models of neonatal brain ischemia [14–16], at different ages [17], in organs different from the brain [6,18], and also in cells different from

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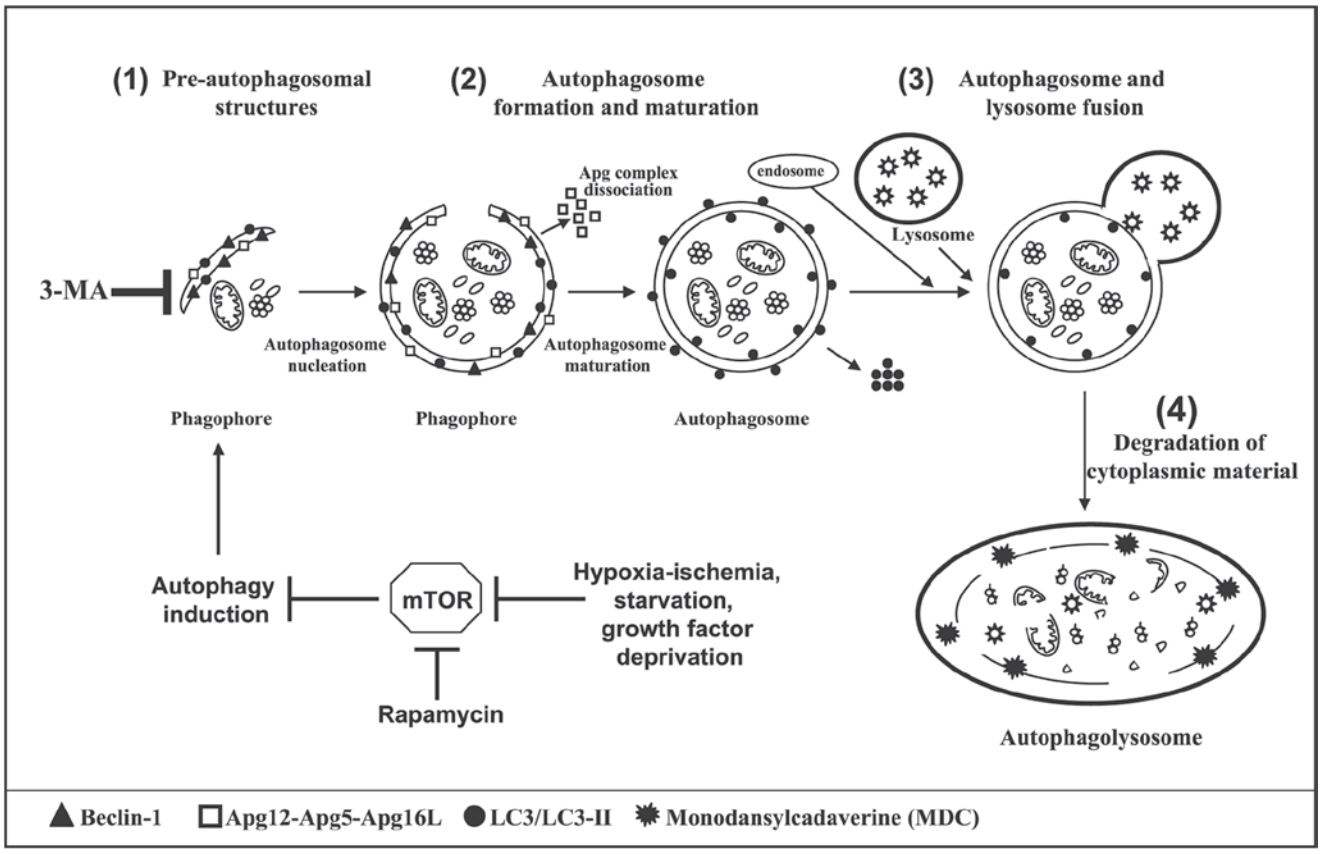

Figure 1. *Model of autophagosome formation in mammalian cells.* A portion of cytoplasm is enclosed by autophagic membrane isolation and will form the autophagosome. The outer membrane of the autophagosome then fuses with lysosomes for bulk degradation. (1) Autophagosomes are generated by elongation of small membrane structures where the Apg12-Apg5-Apg16L complex and LC3 are localized. These proteins play important roles in the elongation and closure of the membrane. Beclin-1, a component of the PI3K complex, is also required in the initial step of autophagosome assembly, although Beclin-1-independent autophagy has been described [45]. 3-MA blocks this initial step of the autophagosome formation. (2) To complete the formation of the autophagosome, the Apg12-Apg5-Apg16L complex dissociates from the membrane and LC3 is lipidated to LC3-II and remains on the autophagosome membrane. (3) Autophagosome–lysosome fusion step. (4) Degradation of cytoplasmatic material inside of autophagolysosome by lysosomal enzymes. MDC is a fluorescent dye that labels acidic endosomes, lysosomes and autophagosomes and is useful to measure *in vivo* autophagic vescicle formation and clearance [20,46]. In the left lower part of the figure is schematically described the role of mTOR as autophagy modulator. Hypoxia-ischemia, starvation or growth factor deprivation reduce mTOR activity and activate autophagy. The same effect can be obtained with Rapamycin that has been identified as a specific inhibitor of this kinase.

neurons [19], indicating that autophagy is a general response occurring in stressed cells. Interestingly, treatments that are neuroprotective in neonatal HI, such as simvastatin or rapamycin, as well as hypoxic preconditioning, further increase autophagy [8,20,21,22].

To add more insight in the effect of autophagy in neonatal HI we studied in more detail the effect of rapamycin which increases autophagy by inhibiting the mammalian target of rapamycin (mTOR). Besides increasing autophagy, administration of rapamycin 30 min before HI also increased the level of phosphorylation of Akt and CREB, two well-known factors implicated in cell survival [23–25]. The over-activation of these survival factors occurred in neuronal cells and was interconnected, since after rapamycin pAkt and p-CREB were over-expressed in the same cells.

Autophagy activation occurs in neuronal cells which concomitantly activate apoptotic pathways

Most autophagy-positive neurons also showed increased caspase-3 activation and were TUNEL-positive. Beclin 1/TUNEL-positive cells were found mainly in the superficial layers of the cerebral cortex – that is the ischemic penumbra where cell death is delayed and there is a strong activation of apoptotic pathways [26,27]. From a morphological point of view, in this area most cells show a continuum from apoptosis to necrosis death phenotypes [28]. Double-labeled staining performed 24 h after HI was used to evaluate if autophagy-positive cells also showed necrotic features. Propidium iodide (PI) is a fluorescent dye utilized in cell culture experiments to stain necrotic cells because it is unable to cross not-damaged lipid membranes. PI has been used *in vivo* to detect necrotic cells after ischemia in adult mice [29] and we set up a method to use this dye after neonatal HI in rats [30]. We found that necrotic cells (PI-positive cells) were present in the damaged side but not in the contralateral one. Twenty-four hours after HI, PI-positive cells were mainly detected in the deep layers of the cerebral cortex and in the hippocampus (the core of the infarct in our model), with only scattered cells in the superficial layers of the cortex. However, at later times (48–72 h after HI) most cells in the superficial layers of the cortex turn out PI-positive (necrotic). Thus, the apoptosis to necrosis continuum can be observed not only morphologically but also by studying biochemical features *in vivo* [30].

What is the role of autophagy activation in ischemic tissues?

To assess the potential role of autophagy in the neurodegenerative process, pup rats were treated before the hypoxic-ischemic

insult with the autophagy inhibitor 3-methyladenine (3-MA) [31]. Twenty-four hours after HI, in 3-MA treated pup rats there was both a marked reduction of beclin 1 expression and a significant increase in the number of necrotic cells in the superficial layers of the cortex. Haematoxylin & eosin staining also showed that 3MA-treated animals exhibited larger areas of necrosis in the cortex with cells showing large pycnotic nuclei [20]. The final morphological injury, however, did not differ from that measured in ischemic untreated rats, suggesting that blocking autophagy does not increase the total number of cells dying as a consequence of the ischemic insult but probably speeds up the transition of the cells that already activated apoptotic pathways towards necrosis.

A similar effect was found when the effect of 3MA was tested on the neuroprotective effect of rapamycin. Indeed, 3-MA blocked rapamycin-induced autophagy and also prevented its neuroprotective effect [20]. Interestingly, WT, which inhibits the PI3K, or the Akt inhibitor IV, a compound that inhibits Akt phosphorylation/activation downstream of PI3K [32], blocked both Akt/CREB phosphorylation and the neuroprotective effect of rapamycin without affecting the activation of autophagy induced by rapamycin. Thus, autophagy is strictly linked to activation of survival factors whose inhibition causes a lack of neuroprotection after drug treatments [20,24,33] or ischemic preconditioning [25,34].

Rapamycin also reduced apoptotic cell death by decreasing the activation of the intrinsic apoptotic mitochondrial pathway since it caused a marked reduction of Bax and Bad translocation to mitochondria, cytochrome c release, and caspase-3 activation [35]. How the anti-apoptotic effect of rapamycin is linked to the strong autophagy signal induced by the drug is not clear. Interestingly, 3-MA administration 10 min after rapamycin restored one of the initial steps that are assumed to trigger the intrinsic apoptotic pathway, i.e. the translocation of pro-apoptotic proteins to the mitochondria. However, after 3-MA the translocation of Bax and Bad to the mitochondria did not cause cytochrome c release and caspase-3 activation, suggesting that the effects of 3-MA might occur upstream of the mitochondria [35]. Furthermore, the reduction of caspase-3 activation by 3-MA did not result in neuroprotection, as could be expected, but gave rise to a rapid transition towards necrotic cell death [20]. Several studies have shown that caspase inhibition does not necessarily prevents cell death [36,37] but can push dying cells towards a necrotic-like phenotype [15,38,39]. Degterev et al. [15] termed the latter phenomena necroptosis, because in this situation cells share the combined biochemical and ultrastructural features of apoptosis and necrosis, and showed that necroptosis proceeded normally also in the presence of 3-MA.

There are contrasting data in the literature on the effect of autophagy on neuroprotection that, overall, add complexity to the pharmacological approach to hypoxia-ischemia induced brain damage. In keeping with our results, Sheng et al. [22], for example, reported that 3-MA pretreatment can suppress the neuroprotective effect of ischemic preconditioning in a model of permanent focal ischemia. These findings indicate that autophagy activation before the hypoxic-ischemic episode could be part of adaptive mechanisms set in motion by hypoxic [22,40,41] or pharmacological preconditioning [8,20] and is associated with activation of survival signaling [20]. Blocking

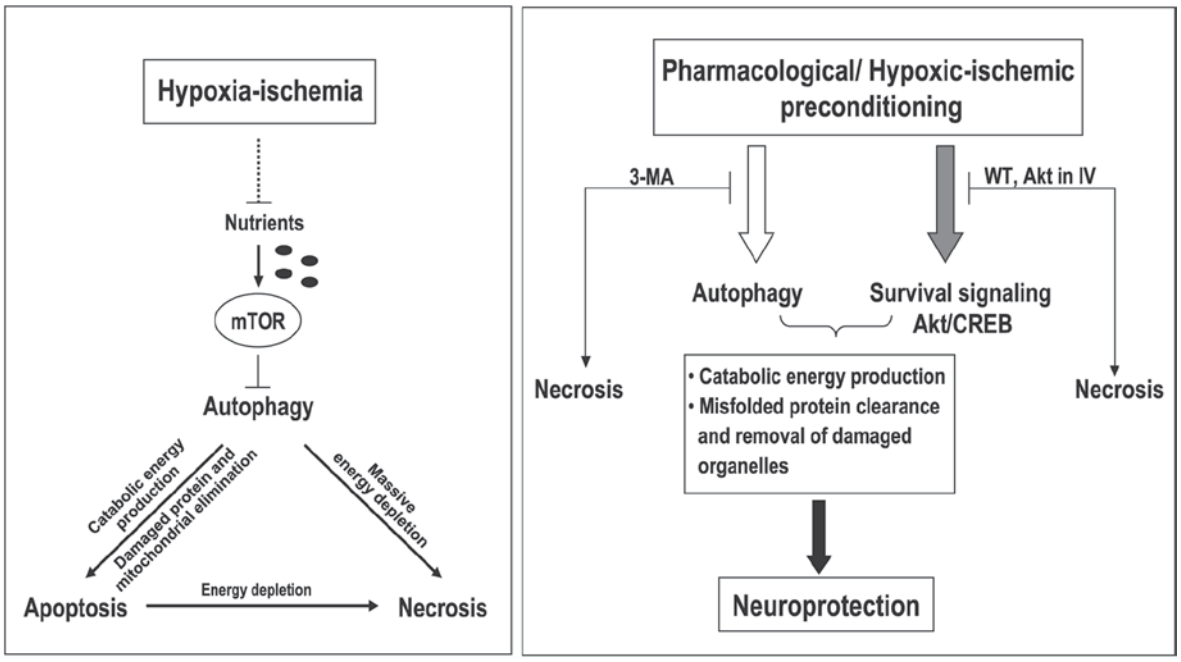

Figure 2. *Schematic overview of the hypothetical role of autophagy in the ischemic penumbra after hypoxia-ischemia and pharmacological or hypoxic/ischemic preconditioning*. According to this scheme, mTOR, the major inhibitory signal that shuts off autophagy in the presence of nutrients, may be a key regulator of autophagy after HI. Ischemia causes nutrient depletion and can activate autophagy by reducing mTOR activity. Autophagy may delay cell death by preserving cellular homeostasis through catabolic energy production and elimination of damaged proteins and mitochondria and by supporting the apoptotic program. This will allow cells to survive longer to the "metabolic stress". Both pharmacological and hypoxic/ischemic preconditioning up-regulate autophagy and may prepare the cells to the "metabolic stress" caused by the ischemic insult (preconditioning-like effect), turning on and sustaining survival programs and postponing the apoptotic program. Either blocking autophagy or pro-survival signals (i.e. Akt and CREB) dramatically increase necrotic cell death, indicating that autophagy and Akt/CREB signals are strictly interconnected. However, a high level of autophagy may also cause massive lysosomal activation and cell death. Because of the strong interplay among autophagy, apoptosis and necrosis, cells may exhibit mixed morphological features of cell death. (WT, wortmannin; 3-MA, 3-methyladenine); Akt in IV, Akt inhibitor IV.

autophagy at this stage is detrimental and speed-up the degenerative process. Puyal et al. [16], in contrast, reported that 3-MA was neuroprotective when administered up to 3 h after reperfusion in a neonatal model of permanent middle cerebral artery occlusion followed by transient left common artery occlusion, and on these basis they claimed that autophagy could be a potential target for novel neuroprotective treatments [42–44]. These divergent results suggest that depending on the contest autophagy can play a dual role: it can be protective when induced before the beginning of the neurodegenerative process but can be detrimental in the later phases. Thus, the final outcome of compounds that block autophagy could be strongly dependent on the timing of administration.

Conclusions

Autophagy is a tightly controlled mechanism and when the supply of macromolecular precursors and oxidizable substrates to maintain viability is low, cells catabolize existing cytoplasmic components to support ATP production to maintain survival. This mechanism appears more complex after ischemia, in which autophagic and apoptotic pathways clearly overlaps. The complexity also originates by the fact that signals that induce apoptosis also increase autophagy, and autophagy pathways are up-regulated also by necrosis-inducing stimuli. Furthermore, activation of autophagy is strictly connected to survival signaling whose inhibition cause cell death (Figure 2). In this complex context, and also in the light of what observed in adult animals and humans where pharmacological treatments to be effective need to be administered before the ischemic insult, it is quite unlikely that a single drug which interferes with a specific cell death pathway could be effective when administered after the hypoxic-ischemic episode. If targeting apoptosis is no more considered a valid approach for treating ischemia because could switch cell death from apoptosis to necrosis, caution is also necessary for using agents that target autophagy because their administration in an early phase of the insult could interfere with endogenous protective mechanisms. On the other hand, also their administration in the late phase of the neurodegenerative process could be challenging because neurodegeneration proceeds slowly and during its progression it is possible that cells in different stages of death pathways activation and nutrients availability may respond in opposite ways to these agents.

Therefore, because of the complex crosstalk between cell death pathways, much effort should be put on the finding of biomarkers that may predict the risk of a hypoxic-ischemic condition in the neonate to initiate the treatment in an early stage, allowing the possibility of using the preconditioning effect of putative drugs. These early treatments may be followed by hypothermia, that potentially reduces both apoptosis and necrosis. Of course, a better understanding of the mechanisms responsible for the switch among the different cell death phenotypes and the development of new and more selective molecules that can act upstream of these putative checkpoints will help to find new pharmacological strategies that could be associated or could be alternative to hypothermia.

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