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·Review·

Neuronal autophagy in cerebral ischemia

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Abstract: Autophagy has evolved as a conserved process for the bulk degradation and recycling of cytosolic components, such as long-lived proteins and organelles. In neurons, autophagy is important for homeostasis and protein quality control and is maintained at relatively low levels under normal conditions, while it is upregulated in response to pathophysiological conditions, such as cerebral ischemic injury. However, the role of autophagy is more complex. It depends on age or brain maturity, region, severity of insult, and the stage of ischemia. Whether autophagy plays a beneficial or a detrimental role in cerebral ischemia depends on various pathological conditions. In this review, we elucidate the role of neuronal autophagy in cerebral ischemia.

Keywords: autophagy; cerebral ischemia; neuron; apoptosis; necrosis

1 Introduction

There are three types of autophagy: macroautophagy, microautophagy[1], and chaperone-mediated autophagy[2]. Macroautophagy is the major form, has been most widely studied, and is best characterized. Therefore, we refer to macroautophagy simply as "autophagy", emphasizing its roles in the survival or death of neurons. Autophagy involves the sequestration of cytosolic components into autophagosomes. Autophagosomes fuse with lysosomes to form autolysosomes, delivering cytosolic contents to the lysosomal lumen, where they are degraded and recycled.

Autophagy plays an important role in the central nervous system, especially in neurons[3-5]. For example, it is important for maintaining homeostasis and protein quality control in the neuron[6]. There is an emerging consensus that the induction of autophagy in neurodegenerative disorders is a neuroprotective response. Inadequate or defective autophagy, rather than excessive autophagy, promotes neuronal death[6-9]. Recently, increased autophagy has been reported in cerebral ischemic injury, including hypoxia-ischemia (HI)[10-19], global[20,21] and focal ischemia[22-30]. However, its role in neuronal death is controversial and it is unclear whether it is beneficial or detrimental. In this review, we summarize the role of autophagy in neuronal death after cerebral ischemia.

2 The autophagic process

To understand the molecular mechanisms underlying ischemia-induced autophagy in neurons, it is crucial to review the details of the regulatory processes. In mam-

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mals, they consist of four principal steps: initiation, vesicle nucleation, vesicle expansion and completion, and vesicle fusion and autophagosome degradation[31].

2.1 Initiation The yeast serine/threonine protein kinase Atg1 and its mammalian homologs ULK1 and ULK2 play critical roles in the initiation of autophagy. ULK1 and ULK2 form a complex with mammalian homolog of Atg13 (mAtg13), the scaffold protein FIP200 (the functional analog of yeast Atg17)[32-34], and Atg101 (an Atg13-binding protein)[35]. Under nutrient-rich conditions, the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) binds to the ULK complex (ULK1/2-mAtg13-FIP200-Atg101). By phosphorylating ULK1 (or ULK2) and hyperphosphorylating mAtg13, mTORC1 inhibits the initiation of autophagy. Under nutrient-deprived conditions, mTORC1 dissociates from the ULK1 complex, freeing it to induce autophagy[32,34]. The inactivation of mTORC1 dephosphorylates mAtg13 and ULK1 (or ULK2), whereas ULK1 and ULK2 still phosphorylate mAtg3 and themselves, and hyperphosphorylate FIP200.

2.2 Vesicle nucleation The activation of the phosphatidylinositol 3-kinase (PI3K) complex is an essential step in vesicle nucleation. In mammals, the PI3K complex is divided into three classes (I, II and III). The class I and III PI3K complexes function as negative and positive regulators of autophagy, respectively. The class III PI3K complex consists of Beclin 1 (a homolog of Atg6), class III PI3K (hereafter referred to as hVps34), and p150 (a homolog of Vps15)[36,37]. Atg14-like protein (Atg14L)[38,39], activating molecule in Beclin-1-regulated autophagy (AMBRA1)[40,41], UV radiation resistance-associated gene (UVRAG) protein[42], and Bax-interacting factor 1 (Bif1)[43] positively regulate autophagy, whereas the RUN domain and cysteine-rich domain containing Beclin-1-interacting protein (rubicon)[38,39] negatively regulate it through the class III PI3K. UVRAG and rubicon also regulate endosome maturation.

2.3 Vesicle expansion and completion Two ubiquitin-like conjugation systems, the Atg12-Atg5-Atg16L1 complex and the LC3 (Atg8 homolog)-phosphatidylethanolamine (PE) conjugate, play important roles in vesicle expansion and completion[44,45]. Atg12 is conjugated to Atg5 by the E1-like protein Atg7 and the E2-like protein Atg10. The Atg12-Atg5 conjugate further interacts with Atg16L, which oligomerizes to form a large complex called the Atg16L complex[45]. LC3 is first cleaved at its C-terminus by the cysteine protease Atg4 to generate cytosolic LC3-I with a C-terminal glycine residue. LC3-I is then activated by the E1-like protein Atg7, and then transferred to Atg3, an E2-like protein specific for LC3, which conjugates LC3 to PE. The lipidated form of LC (LC3-II) is subsequently recruited to the phagophore membrane, whereas the Atg16L complex might act as an E3-like enzyme to catalyze the conjugation[46,47].

2.4 Vesicle fusion and autophagosome degradation Vesicle fusion requires the lysosomal membrane proteins lysosomal-associated membrane protein 1 (LAMP-1), LAMP-2 and the small GTPase Rab7, but the mechanism is less well-characterized[48,49]. After fusion, autophagosome degradation depends on a series of lysosomal/vacuolar acid hydrolases including cathepsins B, D and L. The breakdown products are released back into the cytosol through lysosomal permeases.

3 Autophagy in the brain under basal conditions and during development

In the mammalian brain, there is very little detectable autophagy[50]. Under normal or nutrient-deprivation conditions, the brain is well protected by adaptive mechanisms and glial cells. Therefore, a high level of constitutive autophagy in neurons may not be necessary to maintain the cellular energy needs; indeed, autophagy is not observed in the brain of mice deprived of food for 48 h[50].

Instead, autophagy is critical in the maintenance of neuronal homeostatic functions such as protein and organelle turnover[6]. Insufficient or defective autophagy may be critical in neurodegenerative diseases. Neuron-specific Atg5 or Atg7 knockout causes neuronal degeneration, with accumulation of cytoplasmic inclusion bodies that contain protein aggregates[51,52]. Moreover, autophagy is essential for neuronal development and remodeling; it may support neurite and growth cone remodeling and clear defective

structures in axons and dendrites[41,53,54]. Taken together, these results suggest that constitutively active autophagy at low levels is required to maintain homeostasis and function in the neuron.

4 Autophagy in cerebral ischemia

The first report to demonstrate increased autophagy after cerebral ischemia was that of Nitatori and colleagues[20], who showed that the increased cathepsin B-immunopositive lysosomes in neurons after transient global cerebral ischemia are mostly autolysosomes. Furthermore, the delayed death of CA1 pyramidal neurons after brief ischemia is not necrotic but apoptotic. Since well-established markers for autophagy have not been available, relatively few examples of neuronal death met the necessary morphological criteria of autophagy until recently.

In 2005, Zhu *et al.*[10] were the first to examine the influence of age on apoptotic and autophagic cell death after cerebral HI. These authors showed that the basal level of autophagy, as judged by the autophagosome-related marker LC3-II, is 2.5 times higher in the immature than in the adult brain. After HI, LC3-II levels increase more in the adult than in the immature brain, approximately three-fold higher than in normal controls. In 2006, these authors[11] also examined the influence of sex on autophagy after cerebral HI, but found no sex difference in the induction of autophagy after neonatal HI. Subsequent studies on adult or neonatal cerebral HI have also shown increased expression of LC3-II and increased numbers of autophagosomes in striatal and hippocampal neurons. In other studies, Carloni *et al.*[17] showed that Beclin 1, another marker of autophagy, is significantly increased in the hippocampus and cerebral cortex after HI in neonatal models. These data suggest the involvement of enhanced autophagy in neuronal death following cerebral HI. However, this increase might be due to a defect in lysosomal function causing an accumulation of autophagosomes, or a real increase in autophagic flux, the entire process of autophagy. Therefore, Ginet *et al.*[19] studied the involvement of autophagy using neonatal cerebral HI models and showed that cerebral HI increased not only LC3-II levels but also lysosomal activity including cathepsin D and LAMP-1 in damaged cortical and hippocampal CA3 neurons, demonstrating an increase in autophagic flux.

Similar changes have also been found in models of focal cerebral ischemia. Degterev *et al.*[22] showed an increase of LC3-II in damaged brain tissue after transient middle cerebral artery occlusion (MCAO) in adult mice. Rami *et al*.[23] showed a dramatic elevation of Beclin 1 in neurons in the penumbra after transient MCAO in adult rats. These authors also showed that all cells with upregulated Beclin 1 display dense staining for LC3. Consistent with these observations, further studies demonstrated that focal ischemia enhances both biochemical markers of autophagy and lysosomal activation[26]. Puyal *et al.*[27] showed that temporary MCAO increases LC3-II levels and the numbers of cathepsin D- and LAMP1-positive neurons in neonatal rats. Moreover, double-labeling showed strong punctate autophagosomal (LC3) and lysosomal labeling (cathepsin D and LAMP1) in the same neurons. Liu *et al.*[29] recently also showed that LC3-II protein is up-regulated in the post-ischemic brain after cerebral ischemia/reperfusion. However, they put forward a different opinion that the accumulation of protein aggregate-associated organelles following ischemia is probably due to failure of the autophagic pathway, as a result of lysosome deficiency.

5 Signaling pathways regulating autophagy activation in cerebral ischemia

Although ample evidence demonstrated enhanced autophagy in neuronal death following cerebral ischemia, the signaling pathways regulating its activation remain poorly defined. It is possible that energy-sensing[55], hypoxia, endoplasmic reticulum (ER) and oxidative stress[56] in cerebral ischemia are potent stimuli of neuronal autophagy.

5.1 PI3K-Akt-mTORC1 mTORC, the major inhibitory signal of autophagy, is activated by nutrients (amino-acids), energy (ATP) and growth factors[57]. mTOR exists in two distinct complexes, mTORC1 and mTORC2, that differ in their subunit composition and sensitivity to rapamycin. mTORC1 integrates upstream activating signals that inhibit autophagy through the class I PI3K-protein kinase B

(PKB, also known as Akt) pathway. Class I PI3K is a negative regulator of autophagy that is activated by insulin-like and other growth factor signals. Activation of class I PI3K leads to the phosphorylation of plasma membrane lipids that recruit and activate Akt/PKB by PDK1[58,59]. Then activated Akt/PKB promotes phosphorylation of the protein encoded by tuberous sclerosis protein 2 (TSC2)[60,61]. The phosphorylation blocks TSC2 interaction with TSC1, preventing formation of the TSC1/2 complex, and results in mTORC1 activation[62]. A recent study demonstrated that in neonatal HI, autophagy is part of integrated signaling which includes the PI3K-Alt-mTOR axis[63].

5.2 AMPK-mTORC1 AMP-activated protein kinase (AMPK) is a sensor of cellular bioenergetics, especially in response to energy stress[37]. A decrease in ATP concentration during ischemia increases the AMP/ATP ratio and activates AMPK. Upstream kinases include LKB1 kinase and Ca^{2+}/calmodulin-dependent protein kinase kinase-β (CaMKKβ)[64, 65]. Active AMPK leads to phosphorylation and activation of TSC1/2 and inhibition of mTORC1 activity through Rheb. In mammals, the mTORC1 substrate S6K1 is a positive regulator of autophagy. Recently, it was reported that nicotinamide phosphoribosyltransferase promotes neuronal survival by inducing autophagy *via* regulating the TSC2-mTOR-S6K1 signaling pathway during cerebral ischemia[66].

5.3 Beclin 1-Bcl-2 complex Beclin 1 was identified as a Bcl-2-interacting protein through its BH3 domain[67]. The binding of Bcl-2 to Beclin 1 disrupts the association of Beclin 1 with PI3K, hVps34 and p150, therefore inhibiting autophagy[68]. Intriguingly, only ER-localized, but not mitochondria-localized, Bcl-2 inhibits autophagy[68]. Under stress conditions, Beclin 1 is released and induces autophagy[69,70]. As previously demonstrated, the expression of Beclin 1 in neurons is dramatically increased in neonatal HI or focal cerebral ischemia[17,23]. Ischemia stimulates autophagy through the AMPK–mTOR pathway, whereas ischemia/reperfusion stimulates autophagy through a Beclin 1-dependent but AMPK-independent pathway[71]. Although there are several different mechanisms to regulate the dissociation of Beclin 1 from Bcl-2 during autophagy in mammalian cells[72], the specific mechanism in cerebral ischemia is not yet established.

Hypoxia-inducible factor-1α (HIF-1α) is a key transcription factor activated by the low oxygen conditions during cerebral ischemia. Although HIF-1α stimulates autophagy after cerebral ischemia[73], the underlying mechanism remains unclear. It was reported that HIF-1α stimulates mitochondrial autophagy by the upregulation of BNIP3 in mouse embryo fibroblasts[74]. BH3 domain-containing proteins such as BNIP3, upregulated in models of ischemia-induced delayed neuronal death, might compete with Beclin 1 for binding to Bcl-2 and then release Beclin 1 to stimulate autophagy[75].

5.4 p53 The tumor suppressor and transcription factor p53 has been reported to be pivotal in neuronal apoptosis[76]. Recently, we and other investigators have found a new role for p53 in signaling autophagy[77,78]. p53 induces autophagy through the upregulation of damage-regulated autophagy modulator (DRAM), the p53 target gene encoding a lysosomal protein[77]. In our previous studies, we demonstrated that the NF-κB-regulated p53 pathway also contributes to excitotoxic neuronal death by activating the autophagic process[79]. Overstimulation of N-methyl-*D*-aspartate receptors (NMDARs) induces the upregulation of p53, its target gene DRAM, and other autophagic proteins including LC3 and Beclin 1. Moreover, the NF-κB inhibitor SN50 inhibits the excitotoxin-induced upregulation of p53, its target gene DRAM, and other autophagic proteins.

6 Role of autophagy in cerebral ischemia

Although accumulating evidence indicates that autophagy is enhanced following cerebral ischemia, its functional role in neuronal death is still unclear. Over the years, researchers have used chemical inhibitors or inducers of autophagy to investigate the role of autophagy in cerebral ischemia.

Pretreatment with 3-methyladenine (3-MA) and wortmannin (a PI3K/PLK1 inhibitor), which inhibit autophagy, significantly reduces Beclin 1 expression in the superficial layers of cortex and increases necrotic cell death[17]. In contrast, rapamycin, which increases autophagy, augments

Beclin 1 expression, reduces necrotic cell death, and decreases brain injury. These data suggest that the activation of autophagic pathways is a potential protective mechanism in neonatal HI.

Conversely, in an earlier study, we found that the autophagy inhibitor 3-MA and the cathepsin B inhibitor Z-FA-fmk confer moderate neuroprotection in permanent MCAO models in adult rats by inhibiting the upregulation of LC3-II and cathepsin B, suggesting that an autophagic mechanism contributes to ischemic neuronal injury[26]. In transient focal cerebral ischemia/reperfusion models of neonatal rats, 3-MA also provides substantial neuroprotection even when given >4 h after ischemia[27]. A recent study[21] showed that 3-MA has a time-dependent protective effect on hippocampal CA1 neuronal death after transient global ischemia/reperfusion in young adult rats, and needs to be administered before ischemia. Furthermore, inhibition of cathepsin B release might be another important cause of the protection by 3-MA. Taken together, these data suggest that autophagy activation contributes to neuronal death after cerebral ischemia.

Notably, most chemical inhibitors of autophagy are not entirely specific. For example, 3-MA has potential pro-apoptotic side-effects due to inhibition of the anti-apoptotic PI3K/Akt pathway. A recent study showed a possible pro-autophagic effect of 3-MA[80]. Thus, the use of gene deletions, *in vivo* transgenic/knockout models, or functional knockdown (e.g., with RNAi) are the preferred approaches when possible, because these methods allow a more direct assessment of the resulting phenotype. Deletion of Atg7 protects hippocampal pyramidal neurons after HI injury in Atg7-kncokout neonatal mice[14]. RNAi knockdown of Beclin 1 reduces infarct volume, as well as the histological injury and neurological deficits induced by focal cerebral ischemia in adult rats[30], supporting the conclusion that autophagy plays a pro-death role in acute cerebral ischemia.

Although it remains unclear why autophagy plays different roles in ischemic brain injury, this might depend on brain maturity, region, the severity of ischemia, and the timing of therapeutic interventions. First, the age or maturity of the brain influences autophagic mechanisms after cerebral ischemia. Autophagy is more pronounced in adult than in immature brains, whereas the apoptotic machinery is more pronounced in immature brains[10]. Second, autophagy after cerebral ischemia is region-specific. It is widely accepted that apoptosis and autophagy are energy-dependent and that energy failure causes necrosis. While necrosis predominates in the ischemic core, autophagy and apoptosis often occur at the border of the lesion[23-25]. Third, the extent of neuronal damage and the underlying mechanisms also depend on the severity of the insult. Milder HI insults in neonates are more likely to result in apoptosis than necrosis, whereas severe insults lead primarily to necrotic neuronal death. Following the milder insult, induced autophagy might trigger apoptosis *via* caspase-dependent or -independent pathways[14]. However, after severe insults, limited over-activation of autophagic pathways might delay the progression of cells towards death[17,18]. Lastly, autophagy might play different roles during various stages of cerebral ischemia. It has been reported that autophagy may be protective during ischemia in the heart, whereas it may be detrimental during reperfusion[71]. Autophagy is strongly activated in the brain during reperfusion after cerebral ischemia. Moreover, the inhibition of autophagy is protective, suggesting that autophagy might play a detrimental role during reperfusion[27,28]. However, the role of autophagy during ischemia is still unclear. Our previous study showed the detrimental role of autophagy in fatal ischemia[26]. Interestingly, using the same model, we demonstrated that autophagy activation during cerebral ischemic preconditioning (IPC) confers a remarkable tolerance to subsequent fatal ischemia, and the neuroprotective effects by IPC can be mimicked by autophagy inducers[81]. Thus, the role of autophagy in cerebral ischemia is complex. Whether it promotes cell survival or cell death depends on the pathological situation.

7 Autophagy in the “cell death continuum”

An important feature of neonatal HI is that neuronal death phenotypes are very heterogeneous and cannot be categorized dichotomously as either necrosis or apoptosis[82,83]. Cell death manifests along a continuum from

apoptosis to necrosis with activation of signaling pathways resulting in cell death phenotypes with hybrid structural and biochemical features[84,85]. Portera-Cailiau *et al.*[86] first reported the existence of a "continuum" of apoptotic, necrotic, and overlapping morphologies after excitotoxic injury in the neonatal forebrain. Subsequent studies described the presence of "hybrid" cells with characteristics intermediate between apoptosis and necrosis in various cerebral regions[87,88].

Autophagy is also part of this complex continuum in specific regions following neonatal HI in mice. Koike *et al.*[14] showed that dying pyramidal neurons express both apoptotic and autophagic features following neonatal HI. More recent studies[19] showed that enhanced autophagy in the cortex may be related to apoptosis since some neurons with strong autophagic activity also show apoptotic features. In contrast, neurons in CA1 present only a minimal increase in autophagy but strong apoptotic characteristics. Blocking autophagy inhibits the apoptotic pathway, suggesting that autophagy contributes to programmed cell death by activating the apoptotic pathway[27]. Moreover, Beclin 1-independent autophagy is an important contributor to both the caspase-dependent and -independent components of neuronal apoptosis[89].

Consistent with previous studies, Carloni *et al.*[17] also showed that the autophagic pathway is activated in neurons that show apoptotic features in neonatal HI rat pups. However, these authors showed that inhibition of autophagy switches the cell death continuum from apoptosis to necrosis. Induction of autophagy reduces the progression of cells toward necrotic cell death. This may be due to the role of autophagy in maintaining adequate energy production at the cellular level early after severe HI. Therefore, autophagy could allow apoptosis and delay necrotic cell death by providing energy substrates to cells or through interconnections with apoptosis.

It has also been reported that autophagy is involved in the complex continuum following focal cerebral ischemia. Apoptosis tends to occur late and at the border of the ischemic area, whereas necrosis occurs earlier and at the ischemic core. Around the ischemic core is a region of hypoperfused, electrically silent tissue that barely receives enough blood to keep neurons alive, defined as the "ischemic penumbra". Cell death in the penumbra not only bears a resemblance to necrosis, apoptosis, or a mixture of the two, but also exhibits the biochemical and morphological characteristics of autophagic cell death. Sufficient availability of ATP and intact mitochondrial function are the main determinants to shift apoptosis-doomed neurons away from necrosis[23-25].

Taken together, all of the three cell death morphologies, autophagy, apoptosis, and necrosis, can occur in neurons following cerebral ischemia giving mixed features of cell death. The interactions among autophagy, apoptosis, and necrosis are complex with much crosstalk, and their roles in ischemia need further investigations.

8 Conclusion

In neurons, constitutively active autophagy at low levels is important for maintaining homeostasis and protein quality control under normal conditions. Accumulating evidence has demonstrated increased autophagy in response to cerebral ischemia. However, the role of autophagy is more complex, and depends on brain maturity, region, insult severity and stage of ischemia. Thus, each pathological situation requires a specific study on the role of autophagy. The cell-death morphologies of autophagy, apoptosis, and necrosis can occur in neurons after cerebral ischemia giving mixed features of cell death. A better understanding of the interactions among autophagy, apoptosis, and necrosis might provide new therapeutic targets for the treatment of cerebral ischemic injury.

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