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REVIEW ARTICLE

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A Double-Edged Sword with Therapeutic Potential: An Updated Role of Autophagy in Ischemic Cerebral Injury

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SUMMARY

Cerebral ischemia is a severe outcome that could cause cognitive and motor dysfunction, neurodegenerative diseases and even acute death. Although the existence of autophagy in cerebral ischemia is undisputable, the consensus has not yet been reached regarding the exact functions and influence of autophagy in cerebral ischemia. Whether the activation of autophagy is beneficial or harmful in cerebral ischemia injury largely depends on the balance between the burden of intracellular substrate targeted for autophagy and the capacity of the cellular autophagic machinery. Furthermore, the mechanisms underlying the autophagy in cerebral ischemia are far from clear yet. This brief review focuses on not only the current understanding of biological effects of autophagy, but also the therapeutic potentials of autophagy in ischemic stroke. There are disputes over the exact role of autophagy in cerebral ischemia. Application of chemical autophagy inhibitor (e.g., 3-methyladenine) or inducer (e.g., rapamycin) *in vitro* and *in vivo* was reported to protect or harm neuronal cell. Knockdown of autophagic protein, such as Beclin 1, was also reported to modulate the cerebral ischemia-induced injury. Moreover, autophagy inhibitor abolished the neuroprotection of ischemic preconditioning, implying a neuroprotective effect of autophagy. To clarify these issues on autophagy in cerebral ischemia, future investigations are warranted.

Introduction

Ischemic stroke, often resulted from hypoxic ischemic encephalopathy and acute cerebrovascular accident, is a leading cause of mortality and morbidity worldwide [1]. It could induce severe cognitive and motor dysfunction, neurodegenerative diseases and even acute death [1]. The tissue plasminogen activator (tPA) is the only therapy for acute cerebral ischemia approved by the Food and Drug Administration of United States at present [2,3]. However, the strict 3-h time window for the tPA treatment is the main barrier to acute intravenous thrombolysis [2,3]. Thus, identification or exploration of novel therapeutic targets becomes a major challenge and task in the field.

Many molecular mechanisms, including excitotoxicity, periinfarct depolarization, inflammation, oxidative stress, calcium overload and programmed cell death, contribute to ischemic cerebral damage [4]. The apoptosis and necrosis attributing to the neuronal cell death caused by ischemia have been intensively studied [5]. In recent years, autophagy has been discovered to be an important mechanism adopted by many different types of cells for determining their fate. In cells, autophagy is responsible for degradation of most superfluous proteins and organelles. The cytoplasmic constituents, including organelles, are sequestered into double-membrane autophagosomes, which subsequently fuse with lysosomes where their contents are degraded. Although it is still a topic of debate whether autophagy is a mechanism of cell survival or cell death, the importance of autophagy in various biological and pathological processes is widely accepted [6].

As an intracellular bulk degradation system that is found ubiquitously in eukaryotes, autophagy plays important roles in many physiological processes, including development/differentiation [7–10], immunity [11–14], metabolism [15–19] and aging [20–23], as well as pathophysiological processes, including neurodegeneration [24–28], diabetes [16,29–31], obesity [32,33], cancer [34–38], and inflammation [11,39–41]. Also, autophagic flux has been found in multiple ischemic diseases, such as myocardial infarction [42–44], kidney ischemia [22], liver ischemia [45,46], and cerebral ischemia [47,48]. Generally, in the neuronal system, moderate autophagy is thought to be neuroprotective because autophagy helps to clear aggregated-protein associated with neurodegeneration [49–54]. Inadequate or defective autophagy may lead to neuronal cell death, while excess autophagy, often triggered by intensive stress, can also promote neuronal cell death [47,55]. The existence of autophagy in ischemic stroke has been found for many years; however, it is not sure whether autophagy plays a protective role in ischemic cerebral injury or not yet [56,57]. More and more reports regarding the involvement of autophagy in cerebral ischemic stroke have been published in recent years. These reports have brought attention to the novel recruitment and elaborate regulatory mechanisms of autophagy in cerebral ischemia. Thus, this review paper focuses on the updated role of autophagy in cerebral ischemia and neuronal damage.

Autophagy in Central Nervous System

Autophagy, [from the Greek roots "auto" (self) and "phagy" (eating)] which was first described in 1963, mainly refers to the cellular catabolic processes in which cytoplasmic target material is transported to lysosomes for degradation as an evolutionarily conserved mechanism from yeast to mammals [58]. At least three forms, which include macroautophagy, chaperone-mediated autophagy and microautophagy, have been identified in mammals [58]. The macroautophagy is the most well-studied form of autophagy. In macroautophagy, double-membraned vacuoles are generated, called autophagosomes, which sequester cytoplasmic material before delivering it to the lysosome for degradation [59]. Macroautophagy is regulated by nutrition status and AMP-activated protein kinase (AMPK) [60–63], a sensor of cellular energy. In the process of macroautophagy, several autophagic factors, such as Beclin 1 [64,65], LC3 [66], p62 [67–70], and ULK1 [71–74], regulate the intensity and duration of macroautophagy. Chaperone-mediated autophagy [25,75] involves selective translocation of the cytosolic proteins that are marked by a pentapeptide motif with a consensus sequence similar to KFERQ across the lysosomal membrane, while cytosolic chaperones aid in the target recognition and unfolding. In this process, the lysosomal-associated membrane protein-2α (LAMP-2α) is thought to be rate-limiting for target translocation into lysosomes. Microautophagy, a poorly understood phenomenon in mammalian cells, refers to a process where the lysosome itself takes up small portions of cytoplasm by pinching off a vesicle [76,77]. Because macroautophagy is the major autophagy–lysosomal proteolytic pathway identified in central nervous system [78], the term "autophagy" refers to the macroautophagy in this review. All the above-mentioned observation of autophagy was from animal model and there is no direct evidence of autophagy in ischemic stroke in human yet. However, the autophagy in other neurological diseases, such as Alzheimer's disease, has been showed in human brain tissue [26,79].

Evidence for the Autophagy in Cerebral Ischemic Injury

Accumulating evidence has shown that autophagy is activated in brain tissues or neuronal cells after ischemic stimulation. Individual or combined evidence is provided mainly by morphological observation from electronic microscope, immunohistochemistry, immunofluorescence, and immunoblotting assays.

Evidence from Electronic Microscope

Electronic microscope is widely used in autophagy research and discovered the first autophagy more than 50 years ago [80]. The use of electron microscopy is a valid and important method for observation of changes in various autophagic structures that sequentially form the phagophore, autophagosome, and autolysosome [81]. Electronic microscope validated the first autophagy in the hippocampus CA1 pyramidal neurons after transient global cerebral ischemia in 1995 [82]. It was found that the volume density of cathepsin B-positive lysosomes markedly increased 3 days after ischemic insult, while the autophagic vacuole-like structures also increased at this stage. Adhami et al. [83] reported vacuole associated cortical neurons damage in an adult mouse ischemia-hypoxia model, which ranges from cells harboring multiple cytoplasmic vacuoles to cells completely lacking cytoplasmic contents, suggesting the existence of autophagosomal–lysosomal in neurons in ischemic stroke. Many later documents also reported similar results [84–89]. Electronic microscopy demonstrated that autophagy was not only induced in neurons, but also in glial cells during ischemic stroke [90].

Evidence from Assays of Autophagosomal Marker Proteins

LC3 is a mammalian homolog of the yeast Atg8, which is a specific constituent of the autophagosomal membrane [91]. Therefore, the immunohistochemistry or immunofluorescence of LC3 was used to observe the "punctate LC3" after autophagy, while the immunoblotting is applied to detect the ratio of two forms of LC3 (LC3-II to LC3-I) that could be used to estimate the abundance of autophagosomes. Moreover, Beclin 1, a mammalian ortholog of the yeast Atg6 that is required for autophagosome formation, is also used as a marker of autophagy activation.

Using transgenic GFP-LC3 mice, Adhami et al. [83] found that cerebral ischemia–hypoxia causes redistribution of LC3 proteins. In addition, the difference of LC3 fluorescence intensity between the ischemic and contralateral brain tissue on neonatal or adult rodents has been observed at different time points after hypoxia or ischemia [86,92–95]. Upregulation of Beclin 1 induced by cerebral ischemia was also showed by many reports [48,96,97]. Recently, using *in vivo* imaging technology, Tian et al. [98] showed that autophagic GFP-LC3-positive cells were primarily neurons, not astroglial or microglial cells, and the number of autophagic GFP-LC3 cells was greater in the peri-ischemic area than in the core.

A Double-Edged Sword: The Role of Autophagy in Ischemic Cerebral Injury

Numerous data have demonstrated that autophagy is activated by ischemic insult in various models, and the elevated autophagic activity could be regulated by a wide range of interventions, mainly including pharmacological and genetic methods (summarized in Table 1). There is no question that disrupting the autophagic process in brain is deleterious, particularly for the lifespan of the animal, resulting in the accumulation of dysfunctional or aging macromolecules and organelles [99,100]. However, upon the acute cerebral ischemia stress, whether autophagy plays a beneficial or harmful role in the survival of neuronal cells is not an easy question. Adhami et al. [83] showed for the first time that many damaged neurons displayed features of autophagic/lysosomal cell death, and very few cells completed the apoptosis process in cerebral ischemic stress. This result suggested that the damaged neuronal cells can exhibit multiple forms of cell death morphological features, and autophagy is only one kind of cell death during ischemic injury. Alternatively, autophagy may protect neurons by degrading damaged organelles to abrogate apoptosis or generating energy to delay the onset of ionic imbalance and necrosis after cerebral ischemia–hypoxia. However, these early reports did not determine the exact role of autophagy. Dozens of

Table 1 *In vivo* role of autophagy in cerebral ischemic injury

Animals	Model	Phenotypes	Effect of autophagy in cerebral ischemia injury	References
CD-1 mice	tMCAO	NAD^+ inhibited autophagy	Harmful	105
ICR mice	tMCAO	Edaravone inhibited autophagy	Harmful	113
$CBS^{+/-}$ mice	tMCAO	Tetrahydrocurcumin inhibited autophagy	Harmful	112
$SOD2^{-/+}$ mice	tMCAO	SOD2 knockdown inhibited autophagy	Protective	118
STZ-induced diabetic mice	tCCAO	Autophagy is associated with amyloid-beta generation in diabetic mellitus	Harmful	115
	tCCAO	3-n-Butylphthalide inhibited autophagy	Harmful	116
Adult SD rats	pMCAO	GSK-3 inhibitor enhanced autophagy	Protective	119
	pMCAO	Nampt overexpression enhanced autophagy in early stage	Protective	95
	tMCAO	Hyperbaric oxygen preconditioning enhanced autophagy	Protective	96
	pMCAO	Focal cerebral ischemic preconditioning enhanced autophagy	Protective	127
	4VO	2-methoxyestradiol inhibited autophagy	Harmful	111
	pMCAO	Autophagy inhibitors is neuroprotective	Harmful	102
	tMCAO	Beclin 1 knockdown is neuroprotective	Harmful	89
	tMCAO	β-asarone inhibited autophagy	Harmful	107
	tMCAO	TMEM166 induced autophagy	Harmful	109
	pMCAO	Autophagy inhibitors are neuroprotective	Harmful	103
Adult Wistar rats	tMCAO	GNDF and HGF inhibited autophagy	Harmful	104
Adult Zucker fatty rats	tMCAO	Amlodipine and atorvastatin inhibited autophagy	Harmful	111
Neonatal SD rats	ischemia-hypoxia	Rapamycin enhanced autophagy	Protective	86, 93
	dMCAO	Autophagy inhibitor is neuroprotective	Harmful	88
Neonatal Wistar rats	ischemia-hypoxia	Lithium inhibited autophagy	Harmful	108

SD, Sprague Dawley; 2VO, occlusion of bilateral common carotid arteries; 4VO, 4 vessels occlusion; tMCAO/pMCAO/dMCAO, transient/permanent/distal middle cerebral artery occlusion; tCCAO, transient common carotid artery occlusion; IPC, ischemic preconditioning; SOD2, manganese superoxide dismutase; GNDF, glial gell line-derived neurotrophic factor; HGF, hepatocyte growth factor; Nampt, nicotinamide phosphoribosyltransferase; NAD+, nicotinamide adenine dinucleotide; TMEM166, transmembrane protein 166.

later investigations pointed out the complex effects of autophagy in cerebral ischemia. The autophagy and the controversial impacts of autophagy on cerebral ischemic injury as a double-edged sword have been uncovered.

Detrimental role of Autophagy in Ischemic Cerebral Injury

In 2001, Uchiyama [101] showed that autophagy was induced from the early stage of the glucose–oxygen deprivation in PC12 neuron cells. Administration of autophagy inhibitor 3-methyladenine (3-MA) protected the PC12 cells from apoptosis, indicating that autophagy may lead to neuronal cell death. Mice deficient in Atg7, a necessary catalyst in both conjugation systems for autophagy, showed nearly complete protection from cerebral ischemia-induced caspase-3 activation and neuron death, supporting that autophagy plays an essential role in triggering neuronal death execution after ischemic injury in brain [102]. In addition, focal cerebral ischemia induced by permanent middle cerebral artery occlusion increased the formation of autophagosomes and autolysosomes and expression of LC3-II and cathepsin B [102]. Autophagy inhibitor 3-MA reduced infarct volume, brain edema and motor deficits via inhibiting the ischemia-induced upregulation of LC3-II and cathepsin B [102,103]. Also, in a study in neonatal cerebral ischemia, postischemic intracerebroventricular injections of 3-MA reduced the lesion volume even when given > 4 h after the beginning of the ischemia [88]. RNA interference-mediated downregulation of Beclin 1 inhibited autophagy and attenuated cerebral ischemic injury in rats [89]. Two neurotrophic factors, glial cell line-derived neurotrophic factor (GDNF) and hepatocyte growth factor (HGF), decreased the numbers of LC3-positive cells, suggesting that the protective effects of GDNF and HGF were closely associated with their antiautophagic effects [104]. Moreover, various agents, including NAD^+ [105], propofol [106], β-asarone [107], lithium [108], transmembrane protein 166 [109], ginsenoside Rb1 [110], 2-methoxyestradiol [111], tetrahydrocurcumin [112], edaravone [113], and selenite [114], were reported to decrease ischemic brain damage by blocking autophagy process. In these reports, autophagy caused energy depletion, DNA fragmentation, apoptotic signaling pathways activation and severe damage in intracellular components.

Autophagy is also found to be involved in diseases associated with cerebral ischemia. Zhang et al. [115] showed that exacerbation of ischemia-induced amyloid-beta generation by diabetes might be associated with autophagy activation in mouse brain. Also, 3-n-butylphthalide attenuated amyloid-beta protein generation promoted by diabetes in ischemia through inhibiting abnormally activated neuronal autophagy [116]. Similarly, Zhang et al. [117] demonstrated that neuroprotective effects of amlodipine and atorvastatin in metabolic syndrome model of Zucker fatty rats involved their antiautophagic effect.

Beneficial Role of Autophagy in Cerebral Ischemic Injury

A lot of direct evidence has also been demonstrated on the beneficial role of autophagy in ischemic injury. In focal cerebral ischemia, the brain neuronal cells over-expressing Beclin 1 were found to exhibit damaged DNA but without changes in nuclear morphology, indicating that not all the autophagic cells are predestined to die [48]. 3-MA and wortmannin, two autophagy inhibitors, significantly reduced Beclin 1 expression and switched the mechanism of the cell death mode from apoptosis to necrosis [85,93]. Conversely, rapamycin, which increases autophagy, augmented Beclin 1 expression, reduced necrotic cell death, and decreased brain injury [93], suggesting that inhibition of autophagy might help to switch the mechanism of cell death from apoptotic to necrotic [93]. The same research group provided further evidence showing that the inhibition of Akt/CREB signaling pathway by wortmannin could influence autophagy, and autophagy can be part of an integrated prosurvival signaling, which includes the PI3K-Akt-mammalian target of rapamycin (mTOR) axis [86]. Many other mechanisms were also discovered. SOD2 knockdown exacerbated ischemic brain damage under hyperglycemic conditions via increased oxidative stress and DNA oxidation, which was associated with suppression of autophagy regulators [118]. GSK-3β inhibitor suppressed neuroinflammation by activating autophagy after ischemic brain injury, thus suggesting that GSK-3β is a new target for prevention of ischemic brain injury [119]. Accumulation of p62 under hypoxic stress promotes neuronal cell death, which was partly blocked by autophagy inducer lithium chloride [120], supporting that autophagy promotes neuronal cell survival under hypoxic stress. Melatonin, an antioxidant product, promoted neuron cell survival in glucose–oxygen deprivation, while autophagy inhibitor 3-MA totally blocked the neuroprotection of melatonin [121], suggesting that autophagy is possibly one of the mechanisms underlying neuroprotection of melatonin. Our group demonstrated that induction of autophagy contributes to the neuroprotection of nicotinamide phosphoribosyltransferase (Nampt) in the early stage of cerebral ischemia [95]. Overexpression of Nampt increased LC3 puncta immunochemistry staining, LC3-II/Beclin 1 expression and autophagosomes number both *in vivo* and *in vitro* at 2 h after cerebral ischemia. At the early stage of OGD, autophagy inducer rapamycin protected against neuronal injury induced by Nampt knockdown, whereas autophagy inhibitor 3-MA abolished the neuroprotective effect of Nampt partly. Overexpression or knockdown of Nampt regulated the phosphorylation of mTOR and S6K1 signaling pathway upon OGD stress through enhancing phosphorylation of TSC2 at Ser1387 but not Thr1462 site. All these phenotypes are SIRT1 dependent. Of note, the beneficial effect of autophagy in glial cells, such as astocytes following glucose and oxygen deprivation and focal cerebral ischemia, was also observed [90].

Essential Role of Autophagy in Ischemic Preconditioning and Hyperbaric Oxygen Preconditioning

Ischemic preconditioning is a short period of ischemia followed by a brief period of reperfusion before a sustained ischemic insult, which was found to be a powerful method for limiting cerebral ischemia-induced tissue damage [122]. According to several recent studies, autophagy was believed to play an essential role in ischemic preconditioning-induced protection in many organs [22,45,123,124]. Atg3, an autophagic gene, was found to be upregulated by ischemic preconditioning but downregulated by prolonged ischemia [125], suggesting that the activation of autophagy is a specific response to ischemic preconditioning. In cultured PC12 cells, Park et al. [126] showed that ischemic preconditioning markedly increased LC3-II bands, cathepsin D positive cells, lysosomal activity and autophagic vacuoles, and inhibition of autophagy by 3-MA ameliorated the neuroprotective effects of ischemic preconditioning. This phenotype was confirmed in an *in vivo* rat model of focal cerebral ischemia [127]. Moreover, endoplasmic reticulum (ER) stress inhibitor recovered ischemic preconditioning-induced neuroprotection in the presence of 3-MA [128], suggesting that preactivation of autophagy by ischemic preconditioning can boost endogenous defense mechanisms to upregulate molecular chaperones, and hence reduce excessive ER stress during fatal ischemia. Activation of PI3K-Akt-mTOR axis by autophagy might also be crucial for ischemic preconditioning [129].

Hyperbaric oxygen preconditioning has been used for multiple neurological diseases including ischemic stroke and proved to be a safe treatment in all age and gender groups [130]. The protein expression of LC3-II and Beclin 1 and the formation of autophagosomes were increased by hyperbaric oxygen preconditioning, even higher than those in ischemia. Blockade of autophagy by 3-MA attenuated the neuroprotection of hyperbaric oxygen preconditioning against cerebral ischemia [96].

Issues in the Autophagy-Related Studies on Cerebral Ischemia

In this field, two things are irrefutable. First, autophagy is a fundamental intracellular process and disruption of autophagy in brain for long durations such as knockout autophagy-related genes is detrimental. Second, activation of autophagy in various types of neuronal cells is observed in experimental models of brain injury. Nevertheless, it seems that it is very hard to conduct an unambiguous conclusion on the role of autophagy in cerebral ischemic injury. In our opinion, there may be several issues in the current autophagy-related studies on cerebral ischemia.

One of the key issues is that the used chemical agents are nonspecific to autophagy. For example, it seemed that 3-MA was used widely as an autophagy inhibitor in both *in vitro* and *in vivo* studies. In fact, 3-MA inhibits autophagy via inhibiting PI3K activation [131]. In another word, the observed effects of 3-MA in many studies might not truly reflect the inhibition of autophagy but the inhibition of PI3K-class III. We know that Akt-PI3K signaling indeed critically contributes to autophagy [132–139]; however, Akt-PI3K signaling also has impacts on other biological functions, such as apoptosis and necrosis [140,141]. Thus, the nonspecific effects of 3-MA may not be excluded in autophagy-related researches. In contrast to 3-MA, rapamycin is an autophagy inducer. It has been used to augment autophagy after cerebral ischemia insult to examine the effect of enhanced autophagy on neuronal injury. The proautophagic activity of rapamycin is due

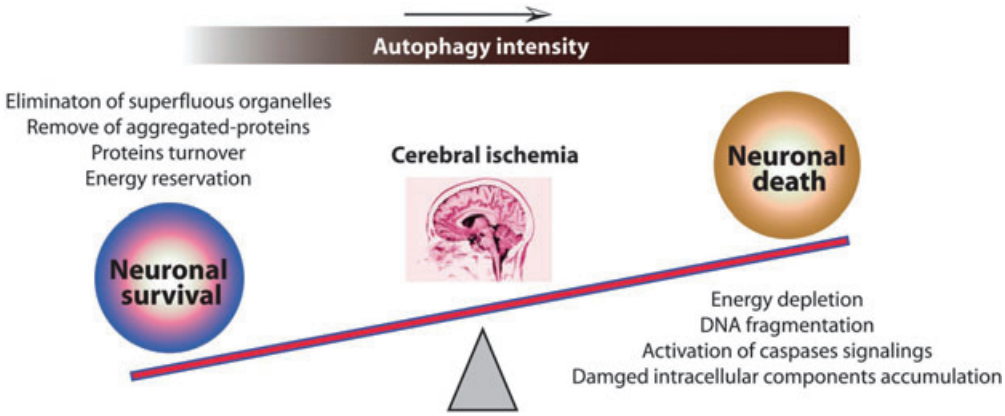

Figure 1 A balance between autophagy-regulated neuronal death and survival in cerebral ischemic stress. Upon cerebral ischemic stress, basal autophagy in neuronal cells acts as a cytoprotective mechanism and serves homeostatic functions such as superfluous organelles removal, aggregated-proteins turnover/elimination and energy reservation to provide metabolic substrates for survival. However, the long-term, uncontrolled and strong, autophagy can digest vital amounts of cell components and survival factors, thus leading to the energy depletion, DNA fragmentation and activation of apoptotic/necrotic signaling pathways, and thereby cell death.

to its inhibitory effect on mTOR, which also results in immunosuppressive and antiproliferative properties [142–144]. Besides the regulatory effect on autophagy, the mTOR plays important roles in growth and metabolism [142,143]. Therefore, it may be hard to separate out these multiple influences in some studies. Therefore, the experimental conditions of inhibitor application and their side effects must be carefully considered.

Another important issue is the reliability of the assays for monitoring autophagy in mouse or rat cerebral ischemia models. Importantly, there are no absolute criteria for evaluating the autophagy activation that apply to every situation. This is because some assays are inappropriate, problematic or may not work at all in particular cells, tissues, or organisms [81,145,146]. There are many acceptable methods to measure macroautophagy in higher eukaryotes summarized in three recent reviews [81,145,146]. Here, we emphasize that no individual assay is guaranteed to be the most appropriate one in every situation, and we strongly recommend the use of multiple assays to monitor autophagy.

Conclusions and Future Perspectives

Although there are disputes over the exact role of autophagy in cerebral ischemia, there is no doubt that autophagy critically contributes to the neuronal fate upon cerebral ischemic stress. How do we reconcile the divergent experimental data on autophagy in ischemic stroke? Cerebral ischemia results in damages to proteins, lipids, and all intracellular components. As a repair mechanism, autophagy is activated to eliminate damaged proteins that accumulate within the neuronal cells. At this stage, autophagy is pro-survival (Figure 1). If the ischemic stress persists for a long time, the autophagy intensity is strengthened consistently. In this case, not only the autophagy is further increased ("supply"), but also the cellular burden of damaged and/or dysfunctional macromolecules and organelles ("demand") is increased (Figure 1). The neuronal cells can not remove all the autophagosomes to return to its basal state, which at last induces neuronal cell death.

However, there are several questions that need to be clarified in the future. Some questions left unanswered are (1) What is the association between autophagy and apoptosis/necrosis during cerebral ischemia? (2) Is there any special autophagic mechanism in neuronal cells triggered by ischemia? (3) Does the autophagy in ischemic/hyperbaric oxygen preconditioning really contribute to the neuroprotection? (4) Can autophagy inducer mimic the ischemic/hyperbaric oxygen preconditioning? To answer these questions is a challenging task!

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Conflict of Interest

The authors declare no conflict of interest.

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