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Review

The divergent roles of autophagy in ischemia and preconditioning

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Autophagy is an evolutionarily conserved and lysosome-dependent process for degrading and recycling cellular constituents. Autophagy is activated following an ischemic insult or preconditioning, but it may exert dual roles in cell death or survival during these two processes. Preconditioning or lethal ischemia may trigger autophagy via multiple signaling pathways involving endoplasmic reticulum (ER) stress, AMPK/TSC/mTOR, Beclin 1/BNIP3/SPK2, and FoxO/NF-κB transcription factors, *etc*. Autophagy then interacts with apoptotic and necrotic signaling pathways to regulate cell death. Autophagy may also maintain cell function by removing protein aggregates or damaged mitochondria. To date, the dual roles of autophagy in ischemia and preconditioning have not been fully clarified. The purpose of the present review is to summarize the recent progress in the mechanisms underlying autophagy activation during ischemia and preconditioning. A better understanding of the dual effects of autophagy in ischemia and preconditioning could help to develop new strategies for the preventive treatment of ischemia.

Keywords: autophagy; ischemic preconditioning; myocardium; endoplasmic reticulum stress; TOR Serine-Threonine kinases; Beclin 1 protein; AMP-activated protein kinases; BNIP3 protein; sphingosines; FoxO proteines

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Introduction

Ischemic heart diseases and stroke are the major causes of death and disability in the world. The strategy in the treatment of ischemic heart diseases and stroke may emerge aiming at regulating endogenous protective mechanisms to reduce the damage and disability associated with ischemia[1–5]. Preconditioning is often a sub-threshold insult applied to the organ that activates certain cellular pathways to reduce damage caused by subsequent severe ischemic episodes[1–5]. Known preconditioning stimuli include inhalational anesthetics, hypoxia, brief ischemia and proinflammatory agents[6]. Recent years, the mechanisms of preconditioning have been systematically studied and several molecular regulatory pathways participating in preconditioning have been elucidated. However, definitive mechanisms underlying preconditioning remain incompletely understood to date[1–7].

Autophagy is an evolutionarily conserved and lysosome-dependent process for degrading and recycling cellular constituents[8, 9]. According to the different pathways by which cellular materials are delivered to the lysosome or vacuole, autophagy is divided into three main categories: macroautophagy, microautophagy and chaperone-mediated autophagy[8]. The major function of autophagy is to remove the bulk of cellular proteins or damaged organelles. Eukaryotic cells have two major intracellular pathways for protein degradation: the ubiquitin-proteasome pathway and the autophagy-lysosome pathway. Short-life proteins are usually degraded via the ubiquitin-proteasome pathway, whereas the long-life proteins and organelles are primarily degraded via the autophagy-lysosome pathway[10].

Recent studies have reported the activation of autophagy following ischemia insult or preconditioning, but the contribution of autophagy to cell death/survival during these processes is still debated.

The debated roles of autophagy in ischemia

In 2006, Adhami *et al*[11] showed that in adult mice with unilateral common carotid artery occlusion and hypoxia, many damaged neurons exhibited features of autophagic/lysosomal cell death with the activation of proapoptosis AIF/caspase signaling pathways. In 2007, Rami[12] reported a dramatic elevation in Beclin 1 and microtubule-associated protein 1 light chain 3 (LC3) levels in the penumbra of rats challenged by cerebral ischemia. A subpopulation of Beclin 1-upregu-

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lated cells also expressed the active form of caspase-3, implying that enhanced autophagy may be a process leading to cell demise. In 2008, Uchiyama's group[13] showed dramatically increased autophagosome formation and extensive death of hippocampal neurons in neonatal mice subjected to hypoxic-ischemic (H-I) injury. A deficiency in the autophagy gene Atg7 provided nearly complete protection from both H-I-induced caspase-3 activation and neuronal death, indicating that Atg7 is a critical upstream gene involved in multiple neuronal death pathways. In addition, our lab provided the first morphological and biochemical evidence in a rodent permanent focal ischemia (pMCAO) model to demonstrate ischemic stroke-induced autophagy activation. Inhibition of autophagy and a lysosomal hydrolase, cathepsin B, significantly reduced ischemic injury and autophagy activation, suggesting that the autophagy-lysosomal pathway is involved in the neuronal death induced by focal ischemia[14]. Like apoptotic cell death, the increased autophagic cell death occurred mainly at the border of the lesion while necrosis occurred at the center of the lesion[15]. Furthermore, neonatal H-I-enhanced neuronal autophagy in the cortex was related to apoptosis, whereas enhanced autophagy in the CA3 was more purely related to the autophagic cell death phenotype. By contrast, neurons in the CA1 presented only a minimal increase in autophagy but strong apoptotic characteristics, suggesting that enhanced autophagy in delayed neuronal death after H-I is differentially linked to apoptosis according to the brain regions[16]. Later, autophagic cell death was also shown to occur in different cell types such as neurons[17, 18], vascular endothelial cells[19] or astrocytes[20] in adult/neonatal focal ischemia and *in vivo* H-I rodent models and in *in vitro* oxygen and glucose deprivation (OGD) models. Inhibition of autophagy, or knockdown of Atg5 or Atg7[18], resulted in protection against neuronal death both *in vivo* and *in vitro*.

However, some studies have suggested that autophagy functions as a protective pathway during ischemia. In 2005, Yan first showed that repetitive myocardial ischemia in pigs triggered autophagy via the increased expression of LC3 and cathepsin B and D[21]. Importantly, they proposed that autophagy could serve as a homeostatic mechanism to inhibit apoptosis and to limit the deleterious effects of chronic ischemia. This viewpoint was further supported by Gottlieb's[22] and Dosenko's[23] group. Ischemia-reperfusion (I-R) of cardiac cells impaired both the formation and downstream lysosomal degradation of autophagosomes. Overexpression of Beclin 1 enhanced the autophagic flux and reduced the activation of pro-apoptotic Bax, whereas inhibition of autophagy via knockdown of Beclin 1, Atg 5 or 3-methyladenine (3-MA) increased cellular injury, suggesting that autophagy constitutes a protective mechanism against I-R or anoxia-reoxygenation injury in cardiac cells. In 2008, Carloni *et al*[24–27] argued that activation of autophagic pathways is a possible protective mechanism in the early stage of the brain ischemia. Beclin 1 was significantly increased in neurons shortly after neonatal H-I, both in the hippocampus and in the cerebral cortex. Autophagy inhibitors switched the mode of cell death from apoptosis to necrosis. Conversely, rapamycin, which enhances autophagy by inhibiting mTOR, reduced both apoptotic and necrotic cell death. Later studies[28] have also shown the protective role of autophagy in renal ischemia. Autophagy was enhanced in kidney tissues during renal ischemia-reperfusion injury. Inhibition of autophagy worsened renal ischemia-reperfusion injury possibly by sensitizing the tubular cells to apoptosis.

Most studies have focused on macroautophagy under ischemia, but there is evidence for the activation of microautophagy and chaperone-mediated autophagy (CMA) after ischemia and hypoxia. At 3–12 h after the induction of pMCAO in rodents, which was accompanied by the formation of autophagosomes, some darkened lysosomes engulfed cytoplasmic materials, implying macroautophagy and microautophagy both were activated[14]. However, the study did not further investigate the role of microautophagy in ischemia. In addition, CMA was also shown to be activated in both hypoxic Neuro2A cells and in the *in vivo* pMCAO model, as evidenced by increased LAMP-2A levels and the accumulation of LAMP-2A-positive lysosomes in the perinuclear area. Blocking LAMP-2A expression with siRNA increased the number of apoptotic cells after hypoxic stress regardless of whether macroautophagy was preserved, whereas administration of the CMA activator reduced hypoxia-mediated cell death, suggesting that CMA is involved in cell survival during hypoxia and ischemia[29].

Interestingly, some studies revealed the distinct roles of autophagy during the ischemia and reperfusion phases of I-R. Autophagy was activated in both the ischemia and reperfusion phases during cerebral I-R. Autophagy plays a detrimental role in permanent ischemia, whereas autophagy during reperfusion may contribute to neuroprotection. Inhibition of autophagy in the reperfusion phase aggravate brain injury induced by I-R[30]. However, these results are in opposition to the study by Matsui *et al*[31]. They showed that in the heart, autophagy provides protection during ischemia, whereas it may be detrimental during reperfusion. These seemly controversial data may be due to the use of different model systems and different signaling pathway involved, but they do suggest that reperfusion may change the role of autophagy during stroke (Table 1).

Autophagy contributes to ischemic tolerance induced by preconditioning

It is generally agreed that autophagy is activated during preconditioning and mediates protection against ischemic insult. Gurusamy *et al* (2008)[32] and Yan *et al* (2009)[33] first showed the cardioprotection induced by ischemic preconditioning (IPC) is associated with autophagy. Preconditioning increased the expression of the autophagy-related protein LC3 and Beclin 1 and promoted the formation of autophagosomes. Inhibition of autophagy by wortmannin abolished the preconditioning-induced cardioprotection. In 2009, a study conducted in PC12 cells[34] first revealed that autophagy is involved in preconditioning-induced neuroprotection. Inhibition of autophagy ameliorated the protection and the upregulation of HSP70

Table 1. The debated roles of autophagy in ischemia. Autophagy has been shown to occur in different cell types, including neurons, vascular endothelial cells and astrocytes, during adult/neonatal hypoxia-ischemia (H-I) injury, transient middle cerebral artery occlusion (tMCAO), permanent middle cerebral artery occlusion (pMCAO), oxygen glucose deprivation-reperfusion (OGD-R), and glucose deprivation (GD) in both *in vivo* and *in vitro* models, although the contribution of autophagy to cell death/survival during these processes is still debated.

Significance	Ischemic model	Cells/area	Selected references
Autophagy contributing to cell death or injury	Mouse H-I	Neurons/cortex	[14]
	Rat tMCAO	Neurons/penumbra	[15]
	Mouse neonatal/adult H-I	Neurons/hippocampus	[16]
	Rat pMCAO	Neurons/cortex & striatum	[17]
	Rat neonatal focal ischemia	Neurons/cortex	[18]
	Rat neonatal H-I	Neurons/cortex & hippocampus	[19, 20]
	In vitro OGD-R	Neurons & SY5Y	[20, 21]
	Mouse pMCAO	Neurons & endothelial cells	[22]
	Rat pMCAO	Astrocytes	[23]
	In vitro OGD	Astrocytes	[23]
Autophagy contributing to protection	Pig chronic myocardial ischemia	Cardiomyocytes	[24]
	Simulated I-R	Cardiac HL-1	[25]
	Anoxia-reoxygenation	Cardiomyocytes	[26]
	Rat neonatal H-I	Neurons/cortex & hippocampus	[27-30]
	Mouse renal I-R	Renal tubular cells	[31]
	In vitro I-R	Renal tubular cells	[31]
	Rat pMCAO	Neurons	[32]
	In vitro hypoxia	Neuro2A	[32]
Autophagy exerting divergent roles in ischemia and reperfusion	Mouse pMCAO/tMCAO	Neurons/cortex	[33]
	In vitro OGD/OGD-R	Neurons/cortex	[33]
	Mouse I-R	Cardiomyocytes	[34]
	In vitro GD	Cardiomyocytes	[34]

that was induced by IPC. In 2010, Gottlieb's group[35] and our lab[36] confirmed that autophagy is associated with IPC-induced protection against myocardial ischemia and cerebral ischemia, respectively, in rodent models. A rapid and significant increase in the number of autophagosomes was found in preconditioned hearts[35]. Cardioprotection by IPC was reduced in hearts perfused with recombinant Tat-ATG5 (K130R), a dominant negative mutation of the autophagy protein Atg5. Furthermore, three structurally unrelated cardioprotective agents, UTP, diazoxide, and ranolazine, induced autophagy in cardiac cells, whereas inhibition of autophagy abolished their protective effects. Our group[36, 37] provided the first evidence that autophagy is involved in preconditioning in rat focal brain IPC and primary cultured cortical neurons OGD preconditioning models. IPC induced remarkable autophagy activation both *in vivo* and *in vitro*. An inhibition of autophagy with 3-MA and bafilomycin A1 abolished the neuroprotection induced by preconditioning, whereas autophagy inducer rapamycin significantly reduced cell injury in lethal brain ischemia, indicating that autophagy activation during preconditioning offers a remarkable tolerance to the subsequent fatal ischemic insult and that IPC's neuroprotection could be mimicked by an autophagy inducer.

Autophagy has also been found to play a protective role in liver ischemic preconditioning[38, 39]. Autophagy was involved in the neuroprotection or cardioprotection elicited by hyperbaric oxygen preconditioning[40, 41] and in hypoxia or anesthetic preconditioning induced by sevoflurane[42, 43] or isoflurane[44].

Interestingly, researchers found that many agents that are known to simulate preconditioning-like effects (pharmacological preconditioning) can also activate autophagy to exert protection against ischemia. The nucleoside adenosine has been shown to mimic both early and late phase ischemic preconditioning. Yitzhaki *et al*[45] found that the A_1 adenosine receptor agonist 2-chloro-N(6)-cyclopentyladenosine (CCPA) caused a significant activation of autophagy in cardiac cells both *in vivo* and *in vitro*. CCPA exerted protection against I-R, but the protection was lost in cells pretreated with an A_1 receptor antagonist or $Atg5K^{130R}$ (a dominant negative autophagy inhibitor), indicating that autophagy plays an important role in mediating the cardioprotection conferred by adenosine or CCPA's pharmacological preconditioning. Autophagy was then demonstrated to be required for the protection by the antimicrobial agent sulfaphenazole[46], the polyphenolic antioxidant resveratrol[47], the enzyme visfatin mediating NAD^+ biosynthesis[48], and the AMPK activator metformin[49]. An inhibition of autophagy abolished the autophagy activation and the ischemic tolerance induced by these agents.

Together, these findings suggest that autophagy is involved in ischemic, hypoxic, anesthetic and pharmacological preconditioning. Autophagy may be a fundamental process that enhances ischemic tolerance.

Divergent roles of autophagy in preconditioning and ischemia

The above studies fully demonstrated the double-edged sword role[50] of autophagy. Excessive autophagy induces cell death via direct autophagic cell death or indirect crosstalk with apoptosis, whereas mild autophagy may remove damaged organelles or abnormal protein to promote cell survival. The divergent roles of autophagy under preconditioning and lethal cerebral ischemia may also be related to different pathological conditions and the time when autophagy is activated or the duration of elevated autophagy activity. Both ischemic preconditioning (IPC) and lethal cerebral ischemia induce autophagy activation, but the extent and persistence of autophagy activation during these two processes are different. IPC induced a mild but prolonged activation of autophagy, in contrast to lethal ischemia, and therefore it may sustain the beneficial effects of autophagic lysosomal degradation while inhibiting the autophagic cell death that occurs during lethal ischemia[51]. Autophagy may also influence different cell signaling pathways in lethal ischemia and preconditioning to regulate cell death and survival, involving interactions with apoptosis and necrosis and the removal of protein aggregates or damaged organelles.

Crosstalk between apoptosis and autophagy

Why is autophagy activated during preconditioning and lethal ischemia but the effects of autophagy under these two pathological states opposite? The dividing line between the dual roles of autophagy remains unclear. The regulation or crosstalk between autophagy and apoptosis may be one of the reasons for the different roles of autophagy in various patho-physiological conditions. Autophagy may contribute to ischemic damage via autophagic cell death or switching cell injury to apoptosis or necrosis. Lysosomes, which degrade the autophagic sequestration, are not only related to necrosis but also involved in apoptosis[52]. Autophagy activation is often accompanied by increased lysosomal hydrolases (cathepsins), whereas cathepsins are involved in caspase activation and the subsequent apoptosis process. Cathepsin B activates caspase-3 via caspase-11 during cell apoptosis[53]. Some reports have suggested that cerebral ischemia may activate autophagy and lysosome cathepsins to trigger apoptosis[54]. The spreading of hydrolytic enzymes from lysosomes into the cytoplasm due to lysosomal membrane injury or rupture has been suggested to occur in brain ischemic injuries or autophagic neuronal death[55, 56]. The neuronal injury induced by permanent focal ischemia or global ischemia-reperfusion can be partly blocked by the autophagy inhibitor 3-MA and the cathepsin B inhibitor Z-FA-fmk[14, 53, 57]. The redistribution of cathepsin B is also inhibited by 3-MA[57].

However, autophagy may also mediate protection during preconditioning by preventing apoptotic or necrotic cell death[24, 26–28, 58]. Inhibition of autophagy in ischemia or preconditioning changes the mode of the cell death to apoptosis or necrosis. Pharmacological preconditioning with the autophagy inducer rapamycin, however, reduced both apoptotic and necrotic cell death by preventing Bax and Bad translocation to the mitochondria during neonatal hypoxia-ischemia.

Elimination of protein aggregates and damaged organelles

The mechanism underlying autophagy's protection during ischemia or preconditioning is also due to the removal of protein aggregates or damaged organelles from post-ischemic neurons. The accumulation of protein aggregate-associated organelles has been observed following ischemia, which is probably due to the failure of autophagy. The resulting protein aggregation on subcellular organelle membranes leads to multiple organelle damage and to delayed neuronal death after cerebral ischemia[59].

In hypoxia and ischemia, the damaged mitochondria may be degraded by mitophagy via the accumulation of PINK1 on the depolarized mitochondria[60]. PINK1 subsequently recruits PARK2/Parkin, an E3 ubiquitin ligase, to the mitochondrial outer membrane. Recent studies have revealed the protective role of mitophagy in ischemia-reperfusion[30] and ischemic preconditioning[61]. Parkin translocates to mitochondria and mediates the mitophagy process during the reperfusion phase of cerebral ischemia-reperfusion. The blockade of autophagy or the specific inhibition of mitophagy during the reperfusion phase reduces mitochondrial clearance and reinforces the ischemia-induced neuronal injury both *in vivo* and *in vitro*. Similar cardioprotective effects of Parkin-dependent mitophagy were demonstrated to contribute to ischemic preconditioning[61]. Parkin knockout was shown to attenuate the preconditioning-induced p62 translocation to the mitochondria and aggravate ischemia-induced cell death.

Signaling to regulate autophagy during ischemia & preconditioning

The information obtained using multiple ischemia or preconditioning model systems shows that the autophagy/lysosomal pathway responds to a variety of internal signal pathways involving endoplasmic reticulum (ER) stress and the AMPK/TSC/mTOR, Beclin 1/BNIP3/SPK2 and FoxO/NF-κB transcription factors to mediate cell survival or death.

Endoplasmic reticulum (ER) stress and autophagy

We believed that the divergent effects of autophagy during ischemia and preconditioning were partly related to endoplasmic reticulum (ER) stress, which includes the unfolded protein response (UPR) initiated by cell stress conditions[62–64]. A major pathway of UPR is the suppression of most protein translations through the phosphorylation of eukaryotic translation initiation factor 2 subunit a (eIf2a) via the PKR-like ER kinase (PERK). Another pathway is the upregulation of the expression of ER-localized molecular chaperones, such as glucose-regulated protein 78 (GRP78/Bip), GRP94, and other molecu-

lar chaperons, such as heat shock proteins (HSPs). However, similar to autophagy, ER stress is a double-edged sword. Although mild ER stress promotes the recovery of ER function and cell survival during subsequent lethal ischemia, prolonged ER stress may activate an ER stress-dependent apoptotic pathway by the induction of caspases-12 and CHOP (C/EBP homologous protein, growth arrest and DNA damage inducible gene 153, GADD153). Mounting evidence has shown that ER stress plays dual roles in the process of fatal cerebral ischemia and preconditioning. Fatal cerebral ischemia induces ER stress characterized by caspase-12 activation[65, 66], whereas preconditioning may upregulate ER molecular chaperones or HSPs to reduce severe ER stress during fatal ischemia[67–69].

Several studies have demonstrated that ER stress contributes to autophagy activation[70, 71]. ER stress induces ER associated protein degradation (ERAD) to monitor and eliminate unfolded/misfolded proteins in the ER. ERAD includes the ubiquitin/proteasome pathway (ERAD I) and the autophagy/lysosomal pathway (ERAD II). When ERAD I is injured, the autophagy/lysosomal pathway functions as ERAD II to degrade abnormal protein aggregates[70]. In yeast, ER stress stimulates the assembly of the pre-autophagosomal structure and formation of autophagosomes and then transports them to vacuoles[71].

A study carried out in a myocardial ischemic-reperfusion (I-R) model showed that induction of autophagy by therapeutic levels of ER stress inducers ameliorated subsequent lethal injury. Importantly, only the autophagy induced with lower doses of the ER stress inducer resulted in a reduction in I-R injury and cardiomyocyte apoptosis, which was accompanied by the induction of GRP78 and Bcl-2. Higher doses of the ER stress inducer, however, were detrimental to the heart and were associated with the induction of CHOP[72]. Consistent with these results, we showed that pre-activation of autophagy by ischemic preconditioning or pharmacological preconditioning upregulated molecular chaperons, including HSP70, HSP60 and GRP78, and hence reduced excessive ER stress-dependent apoptosis during fatal ischemia, as evidenced by downregulation of CHOP, caspase-12 and caspase-3[36]. Moreover, our study further revealed that the ER stress inhibitor salubrinal cancels the protective effects of IPC and inhibits autophagy activation[73]. By contrast, treatment with the ER stress inducers tunicamycin or thapsigargin significantly reduced the cerebral infarct volume and neurological damage induced by lethal cerebral ischemia[74]. Our current studies have focused on elucidating the contribution of the ER chaperone GRP78 in IPC-induced autophagy, and we have found that GRP78 is a key mediator of autophagy activation during preconditioning (unpublished observations). These results strongly support the idea that preconditioning induces mild ER stress and upregulates molecular chaperons, leading to autophagy, which prevents protein aggregation and excessive ER stress-induced apoptosis in subsequent lethal ischemia. Therapeutic doses of autophagy inducers or ER stress inducers may provide a strong justification for the introduction of IPC to treat cerebral ischemia.

Protection due to the AMPK/TSC/mTOR-dependent autophagy pathway

The AMP-activated protein kinase (AMPK) is an evolutional conserved eukaryotic protein kinase perceiving the cellular energy status. During energetic stress or low energy (an increased AMP/ATP ratio), AMPK is activated and phosphorylates TSC2, leading to the inhibition of mTOR (mammalian target of rapamycin), which plays a role in gating the process of autophagy[31]. All the components of AMPK/TSC/mTOR pathways were recently demonstrated to mediate autophagy activation and the subsequent protective effects during ischemia or preconditioning.

The induction of autophagy during ischemia and glucose deprivation was shown to be accompanied by the activation of AMPK and the inactivation of mTOR in cardiac myocytes. The inhibition of AMPK significantly reduced autophagy induction, whereas the survival of cardiac myocytes was decreased by the autophagy inhibitor 3-MA, suggesting that AMPK-dependent autophagy plays a protective role in ischemic cardiac myocytes[31]. Metformin, an activator of AMPK, was recently shown to induce autophagy and to confer protection against subsequent cerebral ischemia[49]. The inhibition of AMPK by compound C or the inhibition of autophagy by 3-MA abolished metformin preconditioning and autophagy activation.

Hamartin, the product of the tuberous sclerosis complex 1 gene (TSC1), is selectively induced by ischemia in hippocampal CA3 neurons but unaffected in CA1 neurons. Suppression of TSC1 expression increases the vulnerability of neurons to cell death following ischemia both *in vitro* and *in vivo*, whereas overexpression of hamartin increases resistance to OGD by inducing productive autophagy through an mTORC1-dependent mechanism[75]. Interestingly, hamartin or TSC1/TSC2-mTOR signaling is also upregulated by IPC or visfatin and protects neurons via the induction of autophagy [48, 58, 75].

mTOR is the catalytic subunit of two functional complexes: mTORC1 and mTORC2. Rictor (the rapamycin-insensitive companion of mTOR) is the component of mTORC2 responsible for the phosphorylation of Akt by binding to mTOR. It is generally believed that mTORC1 inhibits autophagy. Rapamycin specifically inhibits mTORC1, thus inducing autophagy in many cell types. It has been fully demonstrated that rapamycin exerts neuroprotection against cerebral/myocardial ischemia by inducing autophagy[24, 26, 27, 36]. However, mTORC2 may activate autophagy through Rictor. The preconditioning-like effects of resveratrol are associated with autophagy through the differential regulation of mTORC1 and mTORC2[47]. Although resveratrol attenuates the activation of mTORC1, low-dose resveratrol was shown to significantly induce the expression of Rictor, a component of mTORC2, and activate its downstream survival kinase Akt (Ser473). Resveratrol-induced Rictor was found to bind with mTOR, and treatment with Rictor siRNA attenuated the resveratrol-induced autophagy. These results indicate that pharmacological preconditioning may induce autophagy via mTORC1 or the mTORC2-Rictor survival pathway.

These data suggest that drugs acting on the AMPK/TSC/mTOR pathway may be attractive candidates for treating ischemic cardiovascular or cerebrovascular diseases by induction of autophagy.

Dual effects of the Beclin 1/BNIP3/SPK2/BAG1-dependent autophagy pathway

Beclin 1, the mammalian homologue of the yeast Atg6, is an autophagy-associated tumor suppressor. The anti-apoptotic proteins, Bcl-2 and Bcl-XL, bind with the BH3 domain of Beclin 1 to inhibit Beclin 1[76], whereas BH3-only proteins and pharmacological BH3 mimetics induce autophagy by competitively disrupting the interaction between Beclin 1 and Bcl-2 or Bcl-XL[77].

Some reports have implied that Beclin 1-dependent autophagy mediates autophagic cell death during ischemia[12, 31, 78]. A reduction of Beclin 1 expression in cardiac myocytes by RNA interference or urocortin reduced I-R-induced autophagy and enhanced cell survival[78]. An induction of autophagy and cardiac injury during the reperfusion phase was significantly attenuated in Beclin$^{+/-}$ mice[31]. By contrast, Beclin 1-mediated autophagy may also play a protective role in preconditioning. Autophagy elicited by IPC could limit necrosis in injured livers. An increase in Bcl-2 phosphorylation was associated with decreased interactions between Bcl-2 and Beclin 1 and an increased expression of LC3-II, suggesting important roles of Bcl-2 and Beclin 1 in the regulation of autophagy during preconditioning[38].

BNIP3 (Bcl-2/adenovirus E1B interacting protein 3, 19 kDa) is a mitochondrial pro-cell death Bcl-2 family member with a BH3-domain. Studies have suggested that BNIP3-dependent autophagy exerts dual effects on cell survival. Hypoxia upregulates BNIP3 via HIFlα. The upregulated BNIP3 then displaces Beclin 1 from Bcl-2/Beclin 1 or Bcl-XL/Beclin 1 complexes, releasing Beclin 1 and thereby initiating mitochondrial autophagy and decreasing the production of reactive oxygen species[79, 80]. However, BNIP3 overexpression also promotes prolonged autophagy in hypoxic cancer cells, possibly through increased autophagosome-lysosome fusions, leading to autophagic cell death in apoptosis-competent cells[81].

Sphingosine kinase 2 (SPK2) regulates the level of intracellular sphingosine 1-phosphate (S1P) and has been shown to play a role in preconditioning[6]. We showed that SPK2 contributes to isoflurane and hypoxic preconditioning-induced autophagy activation both *in vivo* and *in vitro*. Interestingly, SPK2-mediated autophagy and protection are not S1P-dependent but may be due to its BH3 domain[82], similar to BNIP3. Our results raised a possibility that SPK2 is another BH3-only protein that is upregulated by preconditioning and that can displace Beclin 1 from Bcl-2/Beclin 1 complexes, thus releasing Beclin 1 and initiating autophagy[44].

Proteins of the Bcl-2-associated athanogene (BAG) family function as cochaperones by bridging chaperones to target proteins. By binding with HSP70/HSC70, BAG-1 modulates the chaperone activities of HSP70 and HSC70[83]. Ischemic preconditioning activates autophagy and increases BAG-1 expression. Co-immunoprecipitation and co-immunofluorescence analyses revealed that LC3-II binds with BAG-1. BAG-1 siRNA attenuates the induction of LC3-II and abolishes the cardioprotection achieved by preconditioning, suggesting that BAG-1 is associated with the autophagosomal membrane protein LC3-II and participates in the induction of autophagy via HSC70. Moreover, another BAG family member, BAG-3, is responsible for the induction of macroautophagy in association with HSPB8. Thus, BAG family members are involved in the induction of autophagy for the degradation of damaged or oxidized proteins to promote cell survival[32, 83].

Protein kinase C-dependent autophagy and protection

The protein kinase C (PKC) family of serine/threonine kinases consists of over 10 different isozymes. The mechanism underlying the cardioprotection of sulfaphenazole (SUL, by pharmacological preconditioning) involves a PKC-dependent induction of autophagy[46]. The inhibition of PKC with chelerythrine blocks the activation of autophagy and abolishes the protection mediated by SUL. Recently, PKCθ was shown to mediate ER stress-induced autophagy[84]. The induction of ER stress by thapsigargin or tunicamycin induced PKCθ phosphorylation and translocation to LC3-containing dot structures, and then elicited autophagy, whereas PKCθ activation and stress-induced autophagy were blocked by chelating intracellular Ca^{2+} with BAPTA-AM or by a PKCθ knockdown. Thus, Ca^{2+}-dependent PKCθ activation is specifically required for autophagy in response to ER stress. Moreover, PKCε is the intracellular signal involved in the ischemic tolerance elicited by adenosine and adenosine receptors[85], which has been shown to induce autophagy[45]. Thus, either PKCε or PKCθ may be involved in the process by which preconditioning induces autophagy.

Dual effects of transcription factors NF-κB/FoxO-dependent autophagy

Nuclear factor kappa B (NF-κB) is released from the inhibitory protein IκB and then translocates to the nucleus to initiate gene expression involved in a broad range of biological processes. NF-κB is thought to play dual roles in regulating cell survival and autophagy following an ischemic insult or preconditioning. NF-κB inhibits autophagy-like cell death in ischemia-induced brain damage. Deletion of the NF-κB p50 subunit increases the number of Beclin 1/TUNEL-positive neurons/vascular endothelial cells in the ischemic cortex. The increased autophagic cell death is accompanied by a downregulation of Akt-mTOR signaling. However, the IκB kinase (IKK) plays multiple roles in the induction of autophagy-related genes. Parthenolide, an inhibitor of the IκB kinase (IKK) and NF-κB binding activity, was shown to abolish autophagy activation and to delay the cardioprotection induced by sevoflurane preconditioning[42], suggesting that NF-κB may mediate autophagy during preconditioning. Thus, NF-κB and downstream signaling pathways exert complex regulation activities on autophagy during preconditioning and ischemia.

FoxO transcription factors regulate diverse cellular func-

tions, including differentiation, proliferation, metabolism, and survival. FoxO target genes are also involved in autophagy in cardiomyocytes. Sengupta *et al*[86] reported the differential regulation of the autophagy pathway by FoxO1 and FoxO3 in cultured cardiomyocytes and in mice subjected to starvation or cardiac ischemia-reperfusion. Starvation of cultured cardiomyocytes induces autophagy and promotes FoxO nuclear localization and binding to autophagy pathway gene regulatory sequences. Increased FoxO1 activity preferentially activates Atg12 gene expression, whereas FoxO3 preferentially activates expression of the LC3 gene. Furthermore, in the *in vivo* mouse model, upon starvation, only FoxO3 is activated during the induction of autophagy in ischemia-reperfusion injury. These results provide evidence of an important role of FoxO1 and FoxO3 in regulating autophagy during ischemia-reperfusion. FoxO may also induce the expression of BNIP3, which then displaces the autophagic effector Beclin 1 from inactive Bcl-XL complexes to initiate autophagy, and JNK may be a potent negative regulator of FoxO-dependent autophagy in neurons[87].

In summary, preconditioning or lethal ischemia may trigger autophagy via multiple signaling pathways involving ER stress, AMPK/TSC/mTOR, Beclin 1/BNIP3/SPK2, and transcription factors, *etc* (Figure 1). These signaling pathways may act together in a coordinated manner rather than independently and form a complex network to regulate autophagy activation. The relationship among the distinct signaling pathways involved in autophagy activation as well as the different roles of autophagy during ischemia and preconditioning remains a challenging research topic for future investigation.

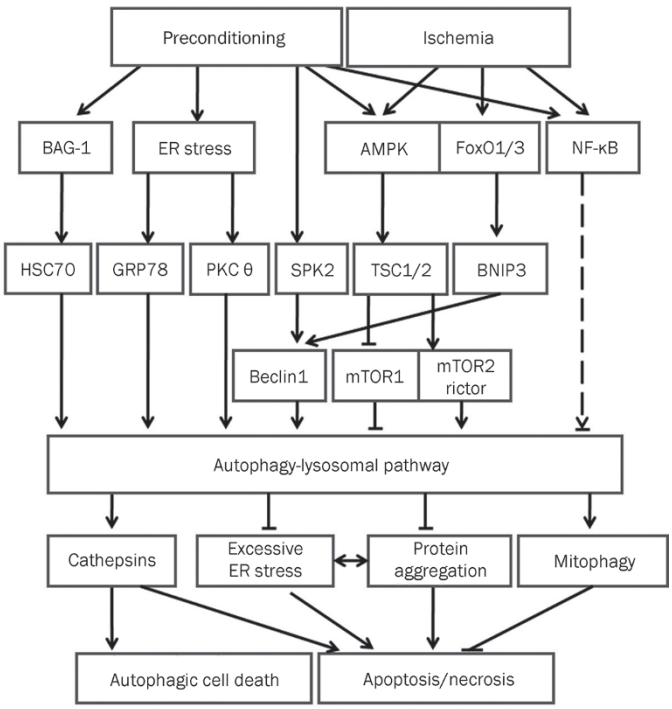

Figure 1. A simplified scheme depicting some of the basic signaling pathways involved in the dual roles of autophagy during ischemia and preconditioning. The autophagy-lysosomal pathway may be activated by endoplasmic reticulum (ER) stress, AMPK/TSC1/2/mTOR, Beclin 1/BNIP3/SPK2 and FoxO and NF-κB transcription signaling. Mild autophagy may remove protein aggregation and damaged mitochondria or relieve excessive ER stress to prevent cell apoptosis or necrosis, whereas excessive autophagy may promote autophagic cell death or apoptosis via cathepsins.

Summary

Autophagy is a double-edged sword. Mild autophagy removes harmful protein aggregates and damaged organelles in cells, thus limiting the spreading of harmful signaling; excessive autophagy, however, induces more irreversible injury in organelles to initiate cell death. The divergent roles of autophagy during preconditioning and lethal ischemia exemplify the double-edged sword roles of autophagy. The present evidence suggests distinct upstream and downstream signaling of autophagy activation during ischemia or preconditioning. Preconditioning or lethal ischemia may trigger autophagy via multiple signaling pathways involving ER stress, AMPK/TSC/mTOR, Beclin 1/BNIP3/SPK2, and transcription factors, *etc*. Autophagy then interacts with apoptosis and necrosis to regulate cell death and survival. Autophagy is also an essential process for maintaining cell function by removing protein aggregates and damaged organelles. However, to date, the double-edged sword roles of autophagy during preconditioning and ischemia have not been fully elucidated. The further development and improvement of molecular biology methodologies and the exploration of the mechanisms underlying autophagy in preconditioning and ischemia will likely provide new avenues for the development of neuroprotective or cardioprotective drugs for the treatment of ischemic cardiovascular disease and a variety of neurodegenerative diseases.

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