Review

The protective roles of autophagy in ischemic preconditioning

Wen-jun YAN^{1,2}, Hai-long DONG², Li-ze XIONG², *

¹Department of Anesthesiology, Gansu Provincial Hospital, Lanzhou 730000, China; ²Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi-an 710032, China

Autophagy, a process for the degradation of protein aggregates and dysfunctional organelles, is required for cellular homeostasis and cell survival in response to stress and is implicated in endogenous protection. Ischemic preconditioning is a brief and nonlethal episode of ischemia, confers protection against subsequent ischemia-reperfusion through the up-regulation of endogenous protective mechanisms. Emerging evidence shows that autophagy is associated with the protective effect of ischemic preconditioning. This review summarizes recent progress in research on the functions and regulations of the autophagy pathway in preconditioning-induced protection and cellular survival.

Keywords: autophagy; ischemia-reperfusion injury; ischemic preconditioning; AMPK; mTOR; Beclin1; P13K; mitochondria; endoplasmic reticulum stress; reactive oxygen species (ROS); apoptosis

Acta Pharmacologica Sinica (2013) 34: 636–643; doi: 10.1038/aps.2013.18; published online 22 Apr 2013

Introduction

Autophagy is an intracellular catabolic process in which the cytoplasmic constituents, such as aggregated proteins and dysfunctional organelles, are surrounded by a double membrane, termed the autophagosome, and are transported to lysosomes for degradation and recycling^[1,2]. Autophagy plays a critical role in cellular development, differentiation and lifespan extension^[2]. Under physiological conditions, the incidence of autophagy is maintained at a low level. However, during various stress conditions, such as oxidative stress, nutrient limitation, hypoxia and endoplasmic reticulum stress, autophagy is induced and is associated with the pathological process of many diseases^[2,3] including cancer, neurodegenerative disorders and ischemic injury. The excessive activation of autophagy can destroy normal proteins and organelles, leading to cell death. This form of cell death is generally termed type-II programmed cell death. However, autophagy also represents an adaptive strategy of the cells. The modest activation of autophagy promotes cell survival. For example, autophagy is activated rapidly during starvation and is responsible for the turnover of unnecessary cytoplasmic proteins and damaged organelles. In this scenario, amino acids and fatty acids are extracted and recycled for the production

of adenosine triphosphate (ATP)^[2].

Ischemic preconditioning (IPC), which is a brief and non-lethal episode of ischemia, confers protection against subsequent ischemia-reperfusion (I/R) through the up-regulation of endogenous protective mechanisms. The IPC-induced protective effect was first identified in the heart by Murry *et al*^[4] and was subsequently found in other organs, such as the brain, spinal cord and kidney^[5,6]. Recent studies have reported that the activation of autophagy is associated with IPC and hyperbaric oxygen-induced ischemic tolerance to I/R injury^[7,8]. In this review, we will discuss the functions and mechanisms of autophagy in the protection induced by preconditioning.

The process of autophagy

Autophagy is highly conserved from yeast to mammals. Depending on the pathways used for the delivery of intracellular cargo to lysosomes, there are three major types of autophagy, macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. Macroautophagy sequesters cytosolic components into double membrane-formed autophagosomes, which fuse with the lysosome for degradation^[9,10]. This degradative process is relatively nonselective and involves the formation, maturation and degradation of autophagosomes. In contrast, chaperone-mediated autophagy is a highly selective degradation pathway that degrades proteins containing the KFERQ-like peptide motif through the lysosomes^[11]. In contrast to macroautophagy and CMA,

* To whom correspondence should be addressed.

E-mail mzkxlz@126.com

Received 2012-12-11 Accepted 2013-02-07

microautophagy involves the direct engulfment of small amounts of cytoplasm by invagination, protrusion, and the separation of the lysosomal membrane^[12], and the process is presumed to depend upon the morphological characteristics of the lysosomes.

Autophagy is divided into four key degradation steps that begin with the formation of the phagophore. The source of the phagophore membrane is likely the endoplasmic reticulum (ER), mitochondria, Golgi complex or plasma membrane^[13, 14]. During phagophore formation, specific proteins, including Atg proteins, are recruited into the forming autophagosomal membranes. This process is regulated by two major functional groups, the Atg9 group and cycling system including Atg9, Atg2, Atg18, and the Atg1 kinase complex (Atg1 and Atg13)^[15], and the functional group consisting of the class III phosphatidylinositol 3-kinase (PI3K) (Vps34), Beclin 1 (yeast Atg6) and p150 (mammalian Vps15)^[16]. The formation of autophagosomes is the second step in the autophagy process and is initiated by the emergence of the phagophore. The phagophore membranes are extended, and they surround the cytosolic constituents to form a double membrane structure termed the autophagosome. The two ubiquitin-like conjugation systems essential for this process are the Atg12-Atg5 system and the microtubule-associated protein 1 light chain 3 (LC3, yeast Atg8)-phosphatidylethanolamine (LC3-PE) system. The Atg12-Atg5-Atg16 complex is localized to the outer membrane of the extending phagophore and is required for the curvature of the extending phagophore^[17, 18]. However, the completion of the autophagosome requires the LC3-PE system. LC3-PE (LC3 II) is recruited specifically to the autophagosomal membrane, is an essential component of the autophagosome^[18] and is used as an autophagic marker. Following the completion of autophagosome formation, the autophagosomes are transported to the lysosomes along microtubules that depend on the dynein-dynactin complex^[19]; therefore, the cytoskeleton plays a role in the maturation of autophagosomes^[20]. The outer membrane of the autophagosome fuses with the lysosome to form the autophagolysosome. During this process, non-Atg components, such as ESCRT, SNAREs, Rab7, and LAMP-2, are required for the fusion of the autophagosome with the lysosome^[21]. In the final step, the contents of the autolysosome are degraded by lysosomal hydrolases, such as lipases and cathepsins; therefore, lysosome activity is essential for functional autophagy. For example, the maturation of the autolysosome requires cathepsin proteases B and D^[22].

Several stress conditions, such as starvation and oxygen deprivation, induce autophagy, which is involved in the protection of organs. Increasing evidence indicates that IPC induces ischemic tolerance to I/R injury through the up-regulation of the endogenous protective mechanism, which is associated with the maintenance of ATP production and intracellular homeostasis, and inhibits apoptotic cell death. These data suggest that IPC and autophagy share many common features, and autophagic pathways play an important role in the protective effect of IPC in I/R injury.

Autophagy pathway mediates cell survival during ischemic preconditioning

AMPK and mTOR

The mammalian target of mTOR is the upstream negative regulator of autophagy in mammalian cells, especially in the absence of nutrients and energy^[23]. During ischemic injury in the heart, the cellular ATP content decreases and AMP accumulates because of energy depletion, which activates the AMP-activated protein kinase (AMPK). The activation of AMPK suppresses mTOR activity, thereby up-regulating autophagy^[24], indicating that the pathway for AMPK-mTOR is important for autophagy regulation. It has been reported that in cultured cardiomyocytes exposed to glucose deprivation, the level of ATP was reduced significantly, leading to the activation of AMPK and suppression of mTOR, which was coincident with the up-regulation of autophagy. The activation of autophagy induced by myocardial ischemia was inhibited in transgenic mice overexpressing a dominant negative AMPK^[25]. It has also been reported that the absence of AMPK aggravated myocardial infarct size in mouse hearts subjected to ischemia, which is associated with autophagosomal formation. These results suggest that the up-regulation of autophagy mediated by the AMPK-mTOR pathway has a beneficial effect on cell survival during ischemia^[24]. AMPK has been shown to contribute to the protective effects of ischemic preconditioning in neurons. Therefore, it is conceivable that autophagy is involved in protection by ischemic preconditioning through the AMPK-mTOR pathway.

Recent studies have demonstrated that AMPK increases the activation of autophagy through the direct activation of Atg proteins, such as Ulk1, rather than through mTOR inhibition^[26]. In addition, there is evidence that p53 suppresses mTOR and subsequently induces autophagy^[27].

Beclin 1

Beclin1, a Bcl-2 interacting protein, is an upstream regulator of autophagy in mammalian cells. In the rat brain, the up-regulation of Beclin1 inhibits the replication of the Sindbis virus and exerts a protective effect on neurons through inhibiting apoptosis^[28], indicating that the activation of autophagy is beneficial to neuron survival. In general, it is accepted that during ischemic injury, autophagy is induced and the expression of Beclin1 is increased. It has been demonstrated that the overexpression of Beclin1 in cultured HL-1 cells protects the cells from ischemic injury. However, the protective effect of Beclin1 was reversed by the expression of a dominant negative mutant of Atg5. Additionally, both down-regulating Beclin1 and activating the Beclin1 domain that binds to Bcl-2 reduced autophagy and increased apoptosis in cardiomyocytes^[29]. These results suggest that the activation of autophagy plays a protective role in cardiomyocytes. Meanwhile, Beclin1 up-regulates autophagy through the direct interaction with Bcl-2, and this has been further demonstrated by Carloni *et al* in hypoxia-ischemia model of the neonatal brain. The study demonstrated that pretreatment with rapamycin, an activa-

tor of autophagy, increased the expression of Beclin1, thereby reducing cerebral ischemia injury and necrotic cell death^[30].

Despite these compelling data, there are controversies concerning the relationship between autophagy and ischemic insult, and there is conflicting evidence that Beclin1-induced autophagy leads to cell death. When the autophagy mediated by Beclin1 was inhibited through the siRNA-mediated down-regulation of Beclin1 expression in cultured cardiomyocytes, the cellular survival increased after ischemic damage^[31]. Mice containing a heterozygous disruption of Beclin1 exhibited an inhibition of autophagy, a decrease in apoptosis and a reduction in infarct size during myocardial ischemia-reperfusion^[24]. However, it is unknown whether the autophagy induced by Beclin1 is detrimental or protective.

PI3K

The class I phosphatidylinositol 3-kinase (PI3K) and class-III PI3K play distinct roles in autophagy. Class-I PI3K inhibits autophagy, whereas class-III PI3K induces autophagy^[32]. For example, 3-methyladenine (3-MA), an inhibitor of class-III PI3K, suppresses autophagy in mammalian cells^[33]. The inhibitory effect of class-I PI3K on autophagy is mediated through the activation of protein kinase B (Akt) following the activation of mTOR^[34]. However, the class-III PI3K forms a complex with Beclin1 and other proteins, which recruits constituent proteins to the autophagosomal membrane, inducing autophagy^[35]. It has been shown that during glucose deprivation in H9c2 cells, the expression of class-I PI3K increased, promoting cardiomyocyte death. Conversely, the treatment with 3-MA and leupeptin, inhibitors of autophagy, inhibited autophagy and reduced cell viability^[36]. There are data demonstrating that rapamycin improves the survival of isolated adult cardiomyocytes exposed to glucose deprivation^[37]. These findings indicate that autophagy enhances cell viability under conditions of energy depletion. In our previous study, we demonstrated that in the focal cerebral ischemia model, rapamycin protects the rat brain from ischemic injury. Furthermore, pretreatment with 3-MA exacerbates the cerebral injury caused by ischemia-reperfusion, suggesting that autophagy-induced neuroprotection against cerebral ischemia may be mediated by class-III PI3K^[8]. It has been reported that the neuroprotective effect of rapamycin in neonatal hypoxia-ischemia is likely associated with the up-regulation of autophagy and activation of the PI3K-Akt-mTOR pathway^[30]. In both the PC12 cell model of oxygen glucose deprivation and the rat brain model of permanent focal ischemia, IPC induced the activation of autophagy, demonstrating that the generation and degradation of autophagosomes was increased after I/R^[38]. However, the inhibition of autophagic activity by 3-MA or Baf A1 attenuated the neuroprotective effect of IPC. Furthermore, rapamycin reduced the cerebral ischemic damage induced by the permanent focal ischemia.

Mitochondria and autophagy

Mitochondria are subcellular units that produce ATP via the oxidative phosphorylation pathway and play an important

role in cell death and survival. Mitochondria undergo continuous remodeling through cycles of fusion and fission to maintain a high functional quality. The damaged and dysfunctional mitochondria that exhibit low membrane potential and generate excessive ROS are degraded through autophagy, namely mitophagy^[39]. A large number of autophagosomes containing mitochondria have been observed during myocardial ischemia-reperfusion injury^[40]. In addition, mitochondria play a role in up-regulating autophagy in cells. For example, a large amount of mitochondrial fragmentation, which occurred prior to the autophagic process, was found in HL-1 cells exposed to ischemia-reperfusion injury^[29]. Recently, it has been demonstrated that mitophagy is up-regulated during ischemic injury^[41]. Because mitochondria release pro-apoptotic factors and cytochrome *c* and produce a large amount of ROS after ischemia-reperfusion, which can lead to apoptosis and necrosis, the up-regulation of mitophagy may have a beneficial effect in ischemic injury^[42].

Several studies have reported that mitochondrial fragmentation and mitochondrial fission are required for inducing mitophagy^[43]. Mitochondrial fission occurred before the up-regulation of autophagy during ischemic injury, whereas the inhibition of mitochondrial fission induced by ischemia reduced the increase in autophagy^[44]. In addition, a study demonstrated that opening the mitochondrial permeability transition pore (mPTP) induced mitophagy after ischemia-reperfusion in cardiomyocytes, whereas inhibiting the mPTP using cyclosporine A, a mitochondrial inhibitor, attenuated the activation of autophagy, suggesting that mPTP acts as an upstream regulator for mitophagy in ischemic injury^[45]. Mitochondrial integrity and the maintenance of energy are important for IPC-induced protection against I/R injury. Purportedly, the mechanism of IPC is associated with the mild depolarization of the mitochondrial inner membrane to open the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) or transiently open the mPTP. It has been proposed that mitochondrial depolarization is a signal for the removal of damaged mitochondria via autophagy. Bnip3, a member of the BH3-only proteins, not only induces apoptosis but also serves as an essential regulator in mitophagy^[46]. Bnip3 triggers a protective stress response with upregulation of autophagy and removal of damaged mitochondria during I/R injury. It has been reported that the up-regulation of mitophagy mediated by hypoxia is associated with the hypoxia-dependent factor-1-dependent expression of Bnip3^[47]. Recent studies have demonstrated that a number of proteins also play a key role in the activation of mitophagy, such as the PTEN-induced putative kinase protein-1 (Pink1) and parkin. It has been reported that parkin induced mitophagy in cells^[48]; meanwhile, parkin was translocated to the mitochondria through Pink1^[49].

The endoplasmic reticulum stress and unfolded protein response

The endoplasmic reticulum (ER) plays a critical role in correct protein synthesis and the folding and maintenance of Ca²⁺ homeostasis. The disturbance of the ER environment or function can induce ER stress, leading to the accumulation

of unfolded or misfolded proteins. ER stress can activate the unfolded protein response (UPR) as a compensatory response, which protect the cells from damage through the activation of multiple functions, such as inducing the degradation of misfolded proteins and the expression of ER chaperone proteins^[50]. Several studies have reported that ischemia-reperfusion activated the UPR in mouse hearts^[51]. The misfolded proteins that accumulate during ER stress are degraded via proteasomes, and excessive unfolded proteins are degraded via autophagy. There is evidence that ER stress induces autophagy. In contrast, the inhibition of autophagy results in cell death mediated by ER stress^[52]. In addition, the activation of autophagy elicited by ER stress is associated with UPR. For example, autophagy was up-regulated by tunicamycin, and thapsigargin acted as an ER stressor in neuroblastoma cells. ER chaperones, such as GRP78 and BiP, also regulate the autophagy induced by ER stress. The knockdown of GRP78 by siRNA inhibited the up-regulation of autophagy induced by ER stress in mammalian cells^[53]. PERK/eIF2 α phosphorylation, an important mediator of the UPR, is involved in the transition of LC3 I to LC3 II in cells^[54]. Moreover, the release of ER calcium was required for the activation of autophagy^[55]. Additionally, ER stress is directly linked to autophagy. It has been shown that Atg1 activity is enhanced when autophagy is induced by ER stress. Furthermore, Beclin1 is localized primarily at the ER^[52]. ER stress is initiated during I/R injury, leading to the accumulation of unfolded or misfolded protein, thereby disrupting cellular homeostasis and resulting in apoptotic cell death. Studies performed on isolated rat heart and primary cultured cortical neurons demonstrated that the increased autophagy induced by IPC suppressed the excessive ER stress during I/R^[56]. 3-MA attenuated neuroprotection by IPC and significantly inhibited the activation of autophagy and ER chaperones, such as GRP78, induced by IPC. Moreover, the ER stress inhibitor recovered the neuroprotection of IPC in the presence of 3-MA. The phosphorylation or activation of eIF2 α and PERK are involved in ER stress in response to I/R injury.

Reactive oxygen species (ROS)

Oxidative stress and increased reactive oxygen species (ROS) are primary mediators of ischemia-reperfusion injury^[57], and in turn, ischemia-reperfusion contributes to the mitochondrial damage that generates excess ROS. Recently, it has been reported that lipopolysaccharide (LPS) treatment enhanced ROS and activated autophagy in neonatal cardiomyocytes^[58]. Furthermore, evadamine, a quinoxaline alkaloid, induced autophagy through the increase in ROS in HeLa cells^[59], which suggests that ROS production is involved in the regulation of autophagy. On the one hand, ROS induces autophagy via mitochondrial damage; a study demonstrated that the inhibition of mitochondrial electron transport chain (mETC) complexes I and II up-regulated autophagy and increased ROS in U87 and HeLa cells. Conversely, treatment with a ROS scavenger inhibited the increased autophagy by inhibiting the mETC complexes^[60]. On the other hand, ROS also

induces autophagy through direct oxidative modifications of the autophagy pathway. For example, H₂O₂ inhibited cysteine protease Atg4 activity, which promoted ATG8-PE conjugation and LC3 conversion, thereby inducing autophagy^[61]. In addition, because Beclin1 expression is dependent on its interaction with Bcl-2, Beclin1 plays a role in the autophagy induced by ROS, which is associated with the expression of Bcl-2. ROS-induced autophagy is associated with cell survival and cell death, which is dependent upon ROS production. In our studies, we have demonstrated that hyperbaric oxygen preconditioning protects the brain from subsequent ischemia-reperfusion injury. The up-regulation of autophagy is involved in the neuroprotection of hyperbaric oxygen preconditioning in a rat model of focal cerebral ischemia^[8]. Furthermore, this neuroprotective effect is mediated by an appropriate increase in endogenous ROS *in vivo* and *in vitro*^[62, 63]. These data suggest that the neuroprotective mechanism of autophagy activation by HBO preconditioning is mediated by the generation of ROS.

Apoptosis and autophagy

In general, it is accepted that autophagy and apoptosis are completely different processes. However, increasing evidence has demonstrated that autophagy and apoptosis are induced by common regulators and signaling pathways. Moreover, cooperation exists between autophagy and apoptosis^[64]. For example, Atg5 not only has an important effect on autophagosome formation but also inhibits apoptosis. It has been reported that the knockdown of Atg5 inhibits autophagy, which induces apoptosis^[65]. Moreover, the inactivation of Atg5 mediated by calpain enhanced cytochrome *c* release and caspase activity^[66], suggesting that the effect of Atg5 inactivation on apoptosis is similar to the effect of the pro-apoptotic proteins belonging to the Bcl-2 family. Additionally, Atg5 interacts with Bcl-X_L, leading to apoptosis, which indicates that Atg5 may promote apoptosis through a secondary apoptotic pathway. In addition, the Bcl-2 binding to Beclin1 also regulates autophagy and apoptosis. It has been reported that the Bcl-2 interaction with Beclin1 inhibits autophagy, and autophagy was inhibited through the overexpression of Bcl-2 in murine heart cells, indicating that Bcl-2 serves as a negative regulator of autophagy through the inhibition of Beclin1^[64]. The BH3 domain of Beclin1 is involved in the binding of Bcl-2 and Beclin1, and a published study demonstrated that the overexpression of a Beclin1 mutant that cannot interact with Bcl-2 induces increased autophagy in the mouse heart^[67]. Therefore, Bcl-2 not only plays an important role in apoptosis but also maintains autophagy levels to promote cell survival rather than cell death. Studies have demonstrated that Bcl-2 negatively regulates the activation of the Beclin1-class III PI3K complex; when the association of Bcl-2 with the Beclin1-class III PI3K complex was interrupted, autophagy was up-regulated in response to stress^[33]. Caspase mediates the cleavage of Beclin1, which induces autophagy; furthermore, a recent study demonstrated that the cleavage of Beclin1 by caspase promoted the production of pro-apoptotic molecules from

Figure 1. Schematic model of autophagy pathways involved in the protective effect of ischemic preconditioning. Ischemic preconditioning causes moderately energy depletion, production of ROS and ER stress, which in turn up-regulate autophagic activity and promote cellular survival. Signaling pathways include activation of AMPK, Beclin1 and class-III PI3K, and inhibition of mTOR and class I PI3K.

mitochondria, leading to apoptosis^[68].

In a cerebral hypoxia-ischemia model of neonatal rat, Carloni *et al* determined that the up-regulation of autophagy plays a neuroprotective role in cerebral ischemia. Beclin1 was markedly enhanced after hypoxia-ischemia and was co-localized with the TUNEL-positive cells, suggesting that the up-regulation of autophagy and promotion of apoptosis occurred simultaneously during ischemic injury. Moreover, inhibiting the activation of autophagy caused rapid cell necrosis by interrupting the progress of apoptosis^[30]. The relationship between autophagy and apoptosis was identified in another study using a rat model of severe perinatal asphyxia^[69]. The expression of Bcl-2 was detected in the liver following IPC, which co-localized with Beclin1. Increased Bcl-2 expression was associated with decreased Beclin 1 and up-regulated LC3-II. In addition, necrosis was reduced by IPC in the liver; however, the TUNEL assay and the expressions of caspase 3, 8, and 9 did not change. These data suggest that the activation of autophagy is involved in the IPC-induced protection against I/R through the interaction of Bcl-2 with Beclin1 and the limitation of necrosis in the human liver^[70].

The studies also demonstrated that 3-MA significantly suppressed the IPC-induced increase in heat shock protein 70 (HSP70) after ischemia, suggesting that the up-regulation of HSP70 participates in the autophagic mechanism underlying the IPC-induced neuroprotection. HSP70 serves as a chaperone and participates in two protein degradation systems, the autophagy/lysosomal pathway and the ubiquitin-proteasomal pathway^[71]. Similarly, the involvement of autophagic activity in ischemic tolerance in IPC has been demonstrated in the heart^[72, 73]. Meanwhile, it has been shown that autophagy

induced by IPC is essential for cardioprotection^[74]. BAG-1 is a multifunctional molecule involved in cell survival that protects cardiomyocytes through the inhibition of apoptosis in association with Hsp70 and the Hsc70 chaperone during I/R injury^[75]. A study demonstrated that the cardioprotection elicited by IPC is mediated by the activation of autophagy in association with BAG-1^[40, 76]. Chemotherapy can aggravate I/R injury during liver surgery; IPC reduced the damage in livers previously treated with chemotherapy. Despite of the classic IPC, there is increasing evidence that agent preconditioning, such as rapamycin and, 2-chloro-N(6)-cyclopentyladenosine (CCPA, an adenosine A1 receptor agonist) mimics the protective effect of IPC in I/R through the significant up-regulation of autophagy^[77, 78]. We demonstrated the essential role for autophagy in a study using a rat model of transient focal cerebral ischemia by middle cerebral artery occlusion; we determined that the activation of autophagy is associated with the neuroprotection of hyperbaric oxygen preconditioning against cerebral I/R injury^[8].

It is conceivable that autophagy is required for the protection elicited by IPC. However, there is an alternative view. For example, Wu *et al* demonstrated that prolonged ischemia induced renal dysfunction in rat through the promotion of apoptosis and autophagy. Following a brief intermittent reperfusion during prolonged ischemia, IPC inhibited the apoptosis and autophagy induced by I/R and reduced renal dysfunction, suggesting that the activation of autophagy plays a negative role in IPC-induced protection^[79]. Further investigations will be required to address this possibility.

Conclusion

Increasing evidence suggests that the activation of autophagy is involved in IPC protection against I/R injury in the body. However, autophagy is a double-edged sword. Excessive autophagic activity can damage normal proteins and organelles resulting in programmed cell death. Therefore, many questions remain regarding the specific mechanism of how and under what conditions the activation of autophagy regulates the protective effect of IPC. Not all factors that induce autophagy are beneficial. For example, intracellular Ca^{2+} overload because of ischemic injury also induces autophagy, which represents damage rather than protection. Moreover, further studies will be needed to determine the optimum autophagic activity needed to obtain maximal benefits during I/R injury. In addition, it has been demonstrated that the activation of autophagy plays an important role in the ischemic tolerance of pharmaceutical or non-ischemic preconditioning to I/R, suggesting that autophagy may be a potential target for the treatment or prevention of organ and tissue injuries by I/R. More studies will be required to elucidate the autophagic cell survival pathways and may allow the clinic application of autophagy for therapeutic purposes.

Acknowledgements

This work was supported by the National Science Foundation for Distinguished Young Scholars (Grant 30725039) and the

key project for the National Natural Science Foundation of China (Grant 30930091).

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