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Review

The role of the autophagy in myocardial ischemia/reperfusion injury ☆

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ABSTRACT

Autophagy is an intracellular process responsible for damaged or unnecessary protein and organelle degradation. In the heart, autophagy occurs at basal level and dysregulated autophagy is associated with a variety of cardiovascular diseases. Autophagy is enhanced in ischemia as well as in the reperfusion phase during cardiac ischemia reperfusion (I/R) injury. More importantly, recent studies revealed that autophagy exerted both beneficial and detrimental effects in pathology of cardiac ischemia reperfusion. This paper is to review the functional significance of autophagy in cardiac ischemia reperfusion injury and discuss underlying signaling pathways. This article is part of a Special Issue entitled: Autophagy and protein quality control in cardiometabolic diseases.

1. Introduction

Autophagy is a cellular process associated with damaged or unnecessary protein and organelle degradation [1]. Previous evidences identified autophagy as a protective intracellular process functioning as protein quality controller and cellular homeostasis keeper. However, a new type of cell death is proposed, namely "autophagic cell death" occurring with autophagy, indicating the complexity of autophagy functions [2,3]. In the heart, autophagy occurs at basal level under normal conditions, contributing to cellular homeostasis through cleaning long-lived or excessive proteins and aged organelles. Deletion of autophagy can result in adverse effects in myocardium [4]. In addition, altered autophagy was observed in many other cardiovascular diseases in response to pathological stimuli, including ischemic heart disease, cardiac hypertrophy and heart failure [5].

There are basically three types of autophagy, namely, macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). These three types differed in ways by which unnecessary components are delivered into lysosomes for final degradation [7]. Macroautophagy, with typical formation of autophagosome, is the most characterized form of autophagy and is better illustrated in mammalian cells. Macroautophagy is responsible for the degradation of both cytoplasmic proteins and intracellular organelles, including endoplasmic reticulum (ER) and mitochondria. Notably, mitochondrial autophagy is also termed as "mitophagy", a process of selective clearance of damaged mitochondria. Mitophagy is now considered as a protective mechanism during cardiac I/R injury, and we will discuss about it in the later part of this review. Apart from the abovementioned three basic types of autophagy, Yuuki Fujiwara et al. reported two novel types of autophagy, termed as "RNautophagy" and "DNautophagy" [8,9]. In these two newly proposed autophagic pathways, RNA or DNA is directly taken in and degraded in lysosomes in an ATP-dependent manner, mainly mediated by the lysosomal membrane protein of $LAMP_2C$. However, the physiological function of RNautophagy or DNautophagy remains unclear.

Acute myocardial infarction (AMI) is one of the major contributors of morbidity and mortality in patients with coronary heart diseases (CHD) worldwide [6]. Under the condition of cardiac ischemia reperfusion injury (I/R injury), the process of autophagy is activated in response to energy crisis and oxidative stress. However, current researches demonstrate that autophagy can be a double-edged sword in the pathological process of I/R injury. In this review, we aimed to discuss the complex functional contribution of autophagy in cardiac I/R injury and identify potential signaling molecules for future clinical development.

2. Molecular signal alternation of autophagy during myocardial infarction and ischemia/reperfusion injury

The heart is comprised of long-lived cardiomyocytes with little regenerative capacity. In myocardium, the self-digestive process of autophagy could occur under normal conditions at a low level, contributing to cellular homeostasis through cleaning long-lived or excessive proteins and aged organelles. Basal level of autophagy is fundamental for cardiac structure and function as for its essential role of protein and organelle quality control. Loss of genes that are essential for

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autophagy could result in cardiac dysfunction and disorders. $LAMP_2$ and Atg5 are essential mediator molecules for autophagy, deficiency of these genes leads to impaired autophagy, subsequent accumulation of abnormal substrates and cardiac dysfunction. Clinical reports observed that mutations of $LAMP_2$, a principle lysosomal membrane protein, were the cause of Danon disease, a condition of severe and progressive myopathy [4]. In addition, K. Nishida et al. reported that Atg5 deficient mice hearts exhibited ventricular dilatation and dysfunction, accompanied by accumulation of damaged proteins and organelles [10].

Altered autophagy is involved in a great number of cardiovascular diseases such as dilated cardiomyopathy, heart failure, anticancer drug-induced cardiomyopathy or ischemic heart disease, autophagy is increased [5]. Decreased autophagic flux activity is observed in glycogen storage disease related cardiomyopathy, for instance, Danon disease is characterized by abnormal accumulation of autophagosome due to defective autophagosome–lysosome fusion. Autophagy is associated with multiple cardiovascular diseases, either as a detrimental contributor to the pathogenesis or as an adaptive response. However, it remains unknown whether the autophagic alteration in these cardiovascular disorders is adaptive or maladaptive. More preclinical studies and clinical analysis should be performed to address this.

2.1. Low ATP level induced AMPK activation upregulated autophagic pathways during cardiac ischemia phase

Cardiac ischemia is characterized by initial restriction of blood supply and low ATP generation, leading to imbalance between blood supply and energy demand, resulting in cardiomyocyte dysfunction and myocardial damage. Adenosine monophosphate-activated protein kinase (AMPK) is a sensitive sensor of cellular energy activated by the decreased level of ATP and high ratio of AMP/ATP under the condition of nutrient deprivation. During the initial phase of ischemia, AMPK was activated by low ATP level in cardiomyocytes. After activation, AMPK regulates the induction of autophagy *via* direct or indirect ULK1 modifications. Previous studies reported that AMPK activated autophagy through AMPK-mTORC1 signaling: AMPK inhibits mTORC1 through phosphorylation of TSC2 and Raptor site, followed by the indirect activation of ULK1. Moreover, recent studies revealed novel pathways through which AMPK activated autophagy. AMPK directly phosphorylates and activated ULK1, enabling the initiation of autophagy [11–13]. The association of AMPK with autophagy during cardiac ischemia was further verified by the finding that autophagy in the initial phase of ischemia was accompanied by AMPK activation and was diminished by dominant negative AMPK [14,15]. AMPK is an essential molecule for autophagy initiation in cardiac ischemia. Joungmok Kim et al. found that AMPK phosphorylation (Ser 317 and Ser 777) is required for ULK1 function in glucose starvation induced autophagy [16,17]. AMPK functions as a nutrient sensor in mediating autophagy machinery in response to energy crisis during cardiac ischemia.

2.2. Hypoxia and HIF-1α also participated in autophagy initiation in cardiac ischemia

Hypoxia-inducible factor 1 alpha (HIF-1α) is a key molecule regulating oxygen homeostasis. It could be activated by low oxygen or increased oxidative stress during cardiac I/R conditions, working potentially as a protective response. *In vitro* studies revealed that HIF-1α mediated mitochondrial autophagy as an adaptive metabolic response under hypoxia conditions in mouse embryo fibroblasts, but the correlation between HIF-1α and autophagy in the disease model of cardiac I/R has not been illustrated [18]. Since previous researches confirmed that HIF-1α activation exerts cardio-protection during I/R, it will be interesting to figure out whether the beneficial effects of HIF-1α against cardiac I/R injury are partly mediated by autophagy in cardiomyocytes.

2.3. Beclin-1 mediated autophagy during cardiac reperfusion phase

Beclin1 (mammalian ortholog of yeast Atg6) plays an essential role in mediating autophagy process, especially in the phase of reperfusion. Since AMPK is no longer activated in reperfusion, it is not the major mediator of autophagy in this phase. Enhanced autophagy during reperfusion is accompanied by upregulation of Beclin1 instead of AMPK, indicating that Beclin-1 protein plays a vital role in autophagy in the phase of reperfusion [14]. Overexpression of Beclin1 increased autophagic activity during I/R *in vitro* [19]. Conversely, depletion of Beclin1 by siRNA transfection or Beclin1 mutation mice attenuated autophagic activity of cardiomyocytes during reperfusion [20].

Beclin1 is a key autophagic protein regulating both autophagosome formation and processing. Collectively, the up-regulation of Beclin1 is responsible for autophagy activation during reperfusion. However, the question on how cardiac I/R injury activates Beclin1 remains to be elucidated. One possible mechanism is its association with Bcl-2 protein. *In vitro* study revealed that Beclin1 mediated autophagic response to nutrient deprivation in cardiac cells is modulated by Bcl-2 protein [21]. In addition, reactive oxygen species (ROS) may also be a strong inducer of Beclin-1 in mediating autophagy during reperfusion [22]. Instead of energy crisis, increased ROS generation is a major promoter of autophagy during reperfusion. Reperfusion phase causes increased oxidative stress and is accompanied by Beclin1 overexpression. Antioxidant MPG intervention significantly suppressed Beclin1 up-regulation, suggesting that ROS may play a key role in mediating Beclin1 up-regulation [22]. Apart from regulating Beclin1 expression, ROS could oxidize and decrease Atg4 activity, contributing to LC3 lipidation and autophagy initiation [23]. Furthermore, as Beclin1 is primarily located in ER, whether ER stress induced by reperfusion conditions also participates in Beclin1 up-regulation needs further exploration.

2.4. "Impaired" autophagic flux in cardiac reperfusion phase

Autophagic flux is a dynamic cellular biological process, from the formation of autophagosome, autophagosome–lysosome fusion to final degradation. It has been deemed that autophagy is further enhanced during cardiac reperfusion phase by providing evidence for accumulated autophagosomes in cardiomyocytes. However, we could not exclude the possibility that the increased formation of autophagosomes during I/R was resulted from decreased autophagosome clearance. Interestingly, X. Ma et al. recently proposed a novel view that autophagic flux was partly "impaired", instead of "excessively activated" during the phase of reperfusion. They found that autophagosome clearance was dramatically decreased with reperfusion in cardiomyocytes, which is detrimental to cardiomyocyte survival during reperfusion [24,25]. This is a novel discovery that is contrary to our traditional view that autophagy is further enhanced during reperfusion phase. This finding also gives rise to the importance of detecting "intact autophagic flux" to reveal the accurate level of autophagy. Additionally, it is of necessity that researchers re-evaluate the previously published results in which pure autophagosome abundance was used to reflect the extent of autophagic activity.

3. Double-edged sword biological function of autophagy in myocardial I/R injury

3.1. Autophagy mediated ATP generation alleviates energy crisis during myocardial ischemia phase

Sufficient ATP supply is an essential requirement for constitutively contrasting cardiomyocytes. However, during the phase of ischemia, ATP generation is decreased due to damaged mitochondrial function and uncoupled phosphorylation. Decreased ATP level is an indirect inducer of cardiomyocyte autophagy *via* AMPK activation. Low ATP level is capable of activating the energy sensor AMPK, followed by up-regulation of AMPK-mTORC1-ULK1 signaling and autophagy

initiation. In the degradation process of autophagy, free fatty acids and amino acids are released, and subsequently recycled to generate ATP through tricarboxylic acid cycle (TCA cycle), compensating for the energy crisis in the condition of cardiac ischemia. It was confirmed that inhibition of autophagy with 3-MA decreased ATP generation and aggravated cardiac cell death in response to glucose deprivation [14]. Cardiac autophagy serves as an energy-recovering process during ischemic phase and is essential for cardiomyocyte survival.

3.2. Mitophagy compensates for mitochondrial injury during cardiac I/R

During the process of cardiac I/R, mitochondrial fission and fragmentation are observed in cardiomyocytes [26,27]. Excessive dysfunctional and damaged mitochondrial during I/R would cause inflammation response and ROS over-production, resulting in myocardial cell death [28]. Under this condition, autophagy is induced to remove the dysfunctional mitochondrial, a process known as mitochondrial autophagy, namely, mitophagy [29,30]. During the initial phase of ischemia, the activation of autophagy functions primarily to maintain energy balance through recovering ATP generation, whereas to switch to damaged organelle or protein clearance in the later phase of ischemia and reperfusion. Undoubtedly, mitophagy is a protective form of autophagy during I/R injury, preventing damaged mitochondrial from releasing cytotoxic substances.

3.3. Autophagy contributes to proteostasis in I/R injury

There are basically two cellular processes responsible for protein quality control through removal of aged or damaged proteins: autophagy for long-lived and macromolecular proteins, while ubiquitin proteasome system (UPS) for short lived proteins [31]. Nevertheless, during the process of I/R, UPS becomes dysfunctional (proteasome activity is preferentially reduced, ubiquitinated proteins exceed the degradation capacity of proteasome), leading to increase in myocardial ubiquitinated proteins and resultant protein aggregates [32]. Unlike UPS, autophagy is activated after I/R, partly functioning to remove these cytotoxic ubiquitinated proteins. Findings by Tannous, P. et al. demonstrated that autophagic activity attenuated protein aggregation in myocardium [33]. Conversely, ubiquitinated proteins were increased in the myocardium of cardiac-specific Atg5-deficient mice [34]. UPS and autophagy work cooperatively in protein quality controlling. Under the condition of cardiac I/R, increased autophagic activity compensated for the impaired UPS function, keeping proteolysis at an appropriate level.

3.4. Detrimental effects of autophagy during reperfusion

Current researches demonstrate that autophagy can be a double-edged sword in the pathological process of I/R injury [35]. Beneficial functions of autophagy during I/R could be attributed to ATP generation and cellular homeostasis, but the underlying mechanisms of detrimental effects of autophagy in reperfusion are still unclear.

Uncontrolled excessive induction of autophagy in response to I/R injury may contribute to autophagic cardiomyocyte death, evidenced by the observation that autophagy suppression by 3-MA or Beclin1 siRNA reduced cell death in cardiomyocyte I/R injury [20]. Autophagy is a cellular process responsible for substrate degradation. If autophagy is hyper-activated beyond a certain level to delete necessary proteins or organelles, leading to cellular dysfunction, autophagic cell death would occur.

Another reason for the detrimental effects of autophagy may be attributed to the crosstalk between autophagy and apoptosis. As Bcl-2 inhibits Beclin1-dependent autophagy, Beclin1 mediated autophagy activation during reperfusion is associated with Bcl-2 down-regulation. Notably, Bcl-2 protein also participates in the regulation of apoptosis. Decreased expression of Bcl-2 may contribute to apoptotic cell death. Another potential molecule that connects autophagy and apoptosis is Bnip3, a pro-apoptotic member of Bcl-2 family. Studies demonstrated that Bnip-3 is involved in autophagy upregulation in cardiac I/R [36]. However, it still remains to be clarified, whether Bnip3 mediated autophagy activation contributes to autophagic cardiomyocyte death, or this autophagy opposes apoptotic cell death pathway.

4. Potential therapeutic targets of autophagy for cardiac I/R injury

It has been a consensus that cardiac autophagy is involved in the pathological progress of cardiac I/R. Therefore, autophagy has become a potential therapeutic interest for researchers. Even though there are no clinical trials targeting autophagy in cardiac I/R treatment, several molecular pathways have been explored in preclinical studies.

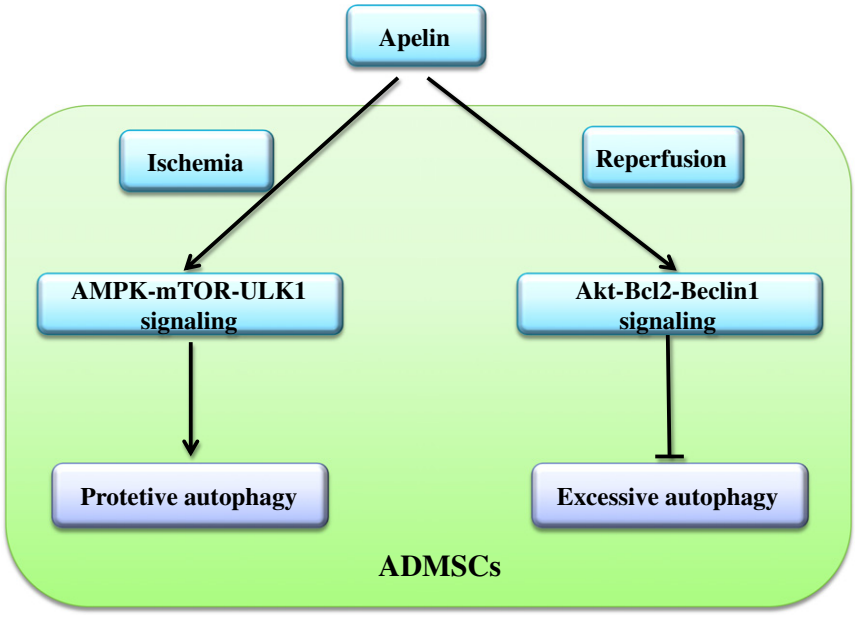

Fig. 1. Mechanisms and functions of autophagy in cardiac ischemia/reperfusion injury.

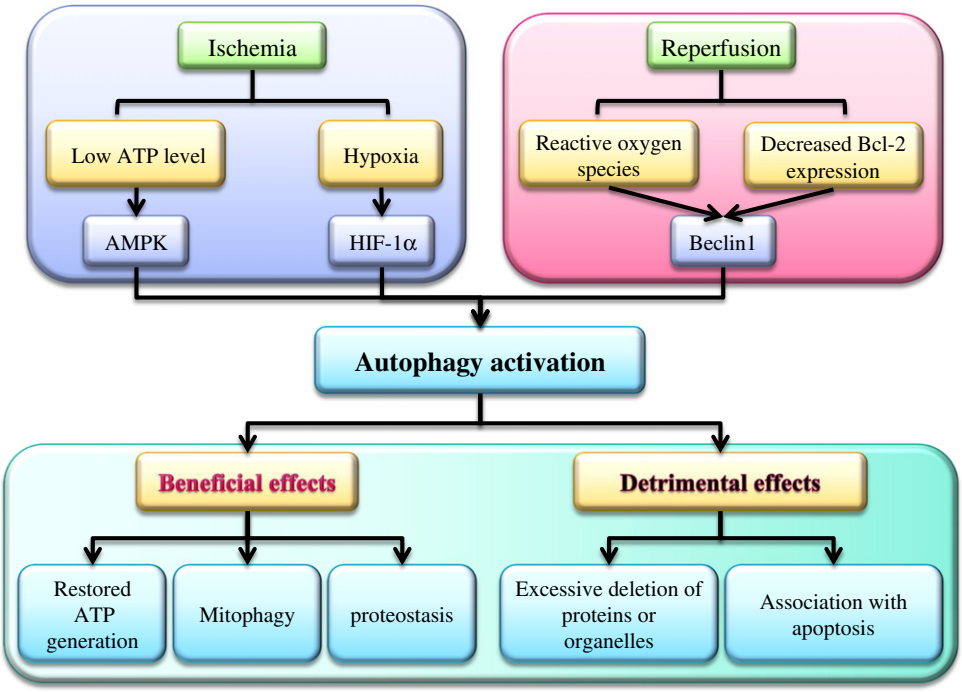

Fig. 2. Dual regulative effects of Apelin in adipose derived mesenchymal stem cell (ADMSC) autophagy in hypoxia–reoxygenation (H/R). Our recent findings indicate that Apelin is capable of dually regulating the activity of autophagy, improving cell viability of ADMSCs. In the phase of initial anoxia, Apelin up-regulated protective autophagy through the activation of AMPK-mTOR-ULK1 pathway, while in the period of reoxygenation, Apelin suppressed excessive autophagic activity through Akt-Bcl2-Beclin1 signaling. The dual regulative effects of Apelin through different signaling mechanisms keep ADMSC autophagy activity at a moderate level to be protective for cell survival.

4.1. AMPK activation could induce autophagy and exert cardioprotective effects against I/R

As increasing lines of evidence have verified that AMPK activation during the initial phase of cardiac ischemia contributes to protective autophagy, pharmacologic drugs for AMPK activation are increasingly attractive as therapeutic targets. *In vitro* evidence showed that AMPK activation by acetylcholine elicited autophagy and induced tolerance against H9c2 cell H/R injury [37]. D942, a cell-permeable compound could also activate AMPK, induce autophagy and exert cardioprotective effects against I/R [38]. A great number of clinical drugs have been shown to activate AMPK, for instance, D942, metformin and stains [39]. These pharmacological compounds may activate AMPK through the regulation of AMP/ATP ratio indirectly. Additionally, Agnes S. Kim et al. reported that a directing AMPK activation *via* small molecule A-769662 significantly alleviated cardiac I/R injury *in vivo* and they demonstrated that pre-treatment of AMPK activation was sufficient to replicate the beneficial function of ischemic preconditioning [40]. Even though AMPK mediated autophagy is protective in cardiac I/R, some concern still remains. One point is that AMPK activation could stimulate fatty acid metabolism while reduce glucose oxidation, resulting to less efficient myocardium metabolism. Moreover, using drugs to activate AMPK systemically may possibly increase food intake by hypothalamus stimuli. Further studies should be directed to understand the feasibility of AMPK activation in cardiac I/R treatment.

4.2. Autophagy regulation through selective activation of mTOR system is associated with enhanced cardiac cell survival after I/R

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase, associating with specific adaptor proteins to form 2 types of multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [41]. Wu, X. et al. reported that valsartan preconditioning significantly reduced cardiac infarction size through autophagy up-regulation *via* PI3K-Akt-mTORC1 pathway [42]. However, Toshinori Aoyagi et al. recently reported that overexpression of cardiac mTORC1 protected the heart from I/R injury [43]. This seems to be a contradiction to our conclusions as mTORC1 overexpression would suppress the pro-survival process of autophagy in myocardium. In fact, the beneficial effects of mTORC1 over-expression are mainly attributed to the suppression of inflammatory response during reperfusion, and the benefits of decreased inflammatory response exceed the functional loss of autophagy. These finding also indicates the complexity of mTORC1 function in a particular disease modal. A specific molecule may be associated with a variety of cellular pathways and have multiple functions. A comprehensive study and evaluation of mTORC1 should be illuminated before clinical usage for autophagy regulator in cardiac I/R patients.

Additionally, we recently uncovered some interesting findings in the regulative function of Apelin on adipose derived mesenchymal stem cell (ADMSC) autophagy in the disease model of hypoxia–reoxygenation (Fig. 1). Similar with cardiac I/R injury, ADMSC autophagy is activated in the initial hypoxia phase and excessively enhanced in the period of reoxygenation. Notably, Apelin is capable of dually regulating the activity of autophagy, improving cell viability of ADMSCs. In the phase of initial anoxia, Apelin up-regulated protective autophagy through the activation of AMPK-mTOR-ULK1 pathway, while in the period of reoxygenation, Apelin suppressed excessive autophagic activity through Akt-Bcl2-Beclin1 signaling. The dual regulative effects of Apelin through different signaling mechanisms keep ADMSC autophagy activity at a moderate level to be protective for cell survival. Our *in vitro* results bring some illumination to the therapies on cardiac I/R injury. It is interesting to find a pharmacological regulator to adjust cardiac autophagy to a moderate extent, thus exerting protective effects during the period of both ischemia and reperfusion.

Interestingly, researchers found that the activation of mTORC2 also participated in the induction of autophagy, and was associated with enhanced cardiac cell survival after I/R [44]. Recently, Völkers, M. et al. proposed a novel approach for cardiac ischemia by selectively increasing

mTORC2 while inhibiting mTORC1 signaling [45]. This indicates that moderate regulation of autophagy, *via* either PI3K-Akt-mTORC1 pathway or mTORC1/mTORC2 balancing, may serve as a potential strategy in cardiac I/R treatment (Fig. 2).

4.3. Other potential agents targeting autophagy for cardiac I/R injury

A series of preclinical studies have revealed the potential cardioprotective benefits of histone deacetylase (HDAC) inhibitors in myocardial I/R model. In a recent study by Min Xie et al., they uncovered that autophagic flux in myocardium infarct border zone was required for the cardio-protective activity of HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA) [46]. SAHA has been approved for cancer treatment by the US Food and Drug Administration. Their study demonstrates that autophagy activation by SAHA pretreatment or SAHA treatment during reperfusion is beneficial for cardiac I/R. More importantly, this study was performed with a preclinical trial in a large-animal of rabbits, using several clinical trial methodologies such as randomization, pre-established end points and strict blinding of personnel. As SAHA serum concentrations in rabbits are achievable in human subjects, SAHA was shown as a potential agent for its control of cardiomyocyte autophagy. However, safety is an important issue that should be addressed. Before it will eventually be applied as a therapeutic strategy in clinical I/R injury, pharmaceutic side effects of SAHA and its molecular mechanisms of its association with cardiomyocyte autophagy should be fully understood.

5. Summary

Currently, the consensus is emerging that autophagy activation during cardiac I/R could either antagonize cardiac pathogenesis or contribute to further myocardium damage, but the underlying mechanism is still unclear. The debate focuses on the detrimental effects of autophagy in I/R. One hypothesis is that excessive autophagy aggravates tissue damage during the period of reperfusion when the heart is load-stressed, accompanied with over-deletion of key organelles or proteins in cardiomyocytes. Besides, some researchers attributed the detrimental effects of autophagy to its association with apoptosis. Several drugs targeting the key molecular of autophagy such as mTOR or Beclin-1 have been applied, for the purpose of alleviating I/R injury in myocardium by regulating the process of autophagy. The key is to suppress excessive autophagy without eliminating basal autophagy, tuning autophagic activity within moderate range for therapeutic benefit in cardiac I/R. In future studies, understanding the function and molecule machinery of autophagy in cardiac I/R injury and exploring potential therapeutic targets of autophagy have significant clinical implications.

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