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Chapter 9

Cell Autophagy and Myocardial Ischemia/Reperfusion Injury

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1. Introduction

Ischemic heart disease is a clinical syndrome resulting from myocardial ischemia and is characterized by an imbalance between the supply and demand of myocardial blood flow and myocardial oxygen metabolism. It is currently one of the major diseases that endanger human health. Early and effective reconstruction of ischemic myocardial blood perfusion is the fundamental measure taken to prevent the development of ischemic myocardial injury, reduce myocardial infarct size, and improve the clinical prognosis. However, several studies have discovered that in some cases, reperfusion of ischemic cells could cause further injury in the form of ischemia/reperfusion injury. The clinical manifestations of myocardial ischemia-reperfusion injury include arrhythmia, myocardial stunning, and no-reflow. Although lethal reperfusion injury in clinical practice is more difficult to identify, it is the most serious consequence of ischemia/reperfusion injury and is also the main reason preventing the ischemic myocardium recovery from effective reperfusion therapy. Therefore, studies on the modes of myocardial cell death after ischemia/reperfusion are of great significance. Previous studies suggested that myocardial cell death following myocardial ischemia/reperfusion injury were mainly necrosis and apoptosis. Apoptosis receives more attention due to its death program. However, in recent years, a number of studies have suggested that, another procedural manner of death---autophagy, type II programmed cell death, also plays a critical role in ischemia/reperfusion injury. The study of this death pathway may provide a new effective way to block myocardial ischemia/reperfusion injury. Therefore, in this chapter, the roles and possible mechanisms of autophagy in myocardial ischemia/reperfusion injury will be reviewed.

2. Definition, formation and classification of autophagy

In 1962, Ashford and Porter discovered the 'self-eating' phenomenon in liver cells using an electron microscope [1]. After that, this process was named autophagy by Duve, a Belgian cellular biologist and chemist [2].

Autophagy is the transportation and degradation of damaged, denatured or aged proteins. It is a common cellular physiological process which can maintain cell homeostasis. Autophagy is an evolutionary conserved pathway of self-digestion that occurs in various eukaryotic organisms from yeast to mammals [3]. It is also a cellular defense mechanism in many pathological processes. The autophagy process time is relatively short (T1/2 for 8 mins), it illustrates that autophagy is an effective response to environmental changes for cells, and it plays a pivotal role in metabolism: (1) Since autophagy is an adaptive response to exogenous stimuli (including nutritional deficiencies, the cell density load, hypoxia, oxidative stress, infection, etc.), and its products of degradation, such as amino acids, nucleotides, and free fatty acids, can participate in the material and energy cycle; (2) As a housekeeper mechanism for cells to maintain a steady state, autophagy can adjust the renovation of long-lived proteins, peroxisomes, mitochondria and endoplasmic reticulum; (3) Autophagy is involved in tissue-specific integration; (4) Autophagy can act as a defense mechanism to remove the damaged cytoplasm and metabolites, and the reconstruction on the subcellular level can protect the affected cells. Meanwhile, as a cell death procedure, autophagy can induce cell initiative death [4].

Although autophagy and autophagy-related processes are dynamic, they can be broken down into several discrete steps. There are four steps in autophagy: induction, formation of autophagosomes, formation of autolysosomes and degradation of the content. Autophagy serves as a response to stress such as nutrient limitation, and this is one of its primary roles in unicellular organisms such as yeast. Then, portions of cytoplasm are first isolated (sequestered) within a double membrane enclosed vacuole called an autophagosome. In studies on yeast, the isolation membrane has been shown to develop from a small vesicle that later transforms into a cup-like structure surrounding the material to be degraded [5]. The formation of an autophagosome (sequestration) is complete when the edges of the 'cup' merge. It has been found that the proteins forming the isolation membrane in yeast are unique, different from those in other cellular compartments, which suggests a de novo formation of this membrane [6]. It is still discussed whether the isolation membrane also forms de novo in higher organisms, including mammals, or if it originates from another organelle, such as endoplasmic reticulum, lysosome or Golgi complex. The sequestration membrane that later gives rise to the autophagosome has been termed phagophore or pre-autophagosome [7]. In yeast and mammalian cells, autophagy occurs at a basal level. The general diameter of autophagosomes is 300-900 nm, and the average level is 500 nm. Since the beginning of the formation of the autophagosomes, cytoplasm and nucleoplasm become darker, but the nucleus structure has no noticeable changes. Mitochondria and endoplasmic reticulum swell, Gorky body expands, and then the membrane-specialized structures such as microvilli disappear, membrane foamed and retracted. At the later stages of autophagy, the volume and number

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of autophagosomes filled with myelin or liquid increase, and then some gray ingredients and a small number of condensated nuclear materials will exist. These features can be used as morphological indicators during inspection.

Depending on the different ways cellular material is transported to lysosomes, there are three types of autophagy: microautophagy, macroautophagy, chaperone-mediated autophagy (CMA). Macroautophagy is commonly referred to as autophagy, and the cytoplasm is wrapped by the dropped bilayers from the non-ribosomal region of endoplasmic reticulum, the Golgi apparatus and other bilayers. In microautophagy, it also goes through the same process of wrapping, but the substrate is engulfed by inward invagination of the lysosomal membrane. In the process of CMA, intracytoplasmic proteins are bound to the molecular chaperone, then transported to the lysosome cavity, and digested by the lysosomal enzyme. CMA sequester proteins that expose a KFERQ-like pentapeptide which are mediated by heat shock cognate 70 (HSC70) and its co-chaperones. Lysosomal-associated membrane protein 2 (LAMP-2A) acts as a receptor on the lysosome and mediates the degradation of unfolded proteins. Macroautophagy is the major regulated cellular pathway for degrading long-lived proteins and the only known pathway for degrading cytoplasmic organelles (Figure 1). Therefore, this chapter focuses on macroautophagy.

3. Signaling regulation of autophagy

Autophagy is highly conservative during the evolution process. Homologous gene participation in autophagy not only be found in yeasts and Drosophila melanogaster but also in vertebrates and humans. In order to unify the standard, Klionsky, in 2003, named these homologous genes as autophagy-related genes (Atg) to stand for autophagy genes and the corresponding proteins [8]. So far, scientists have already found more than 30 Atgs and most of their homologous analogues. In addition, the Atg protein can be divided into five groups: Atg1 protein kinase complex, Atg9, the class III phosphatidylinositol 3-kinase (PI3K)-Beclin1complex, Atg12 conjugation system and Atg8 conjugation system. Atg1 protein kinase complex is essential for the induction of autophagy. There are two mammalian homologs of Atg1 that appear to function in autophagy, the Unc-51-like kinase 1 (ULK1) and -2 (ULK2), which is the mammalian homologues of serine/threonine protein kinase Atg1, existing in the form of ULK1-mammalian Atg13 (mAtg13) -focal adhesion kinase family interacting protein of 200 kDa (FIP200) -Atg10 complex [9]. Under nutritional deficiency conditions, ULK1 is activated and then phosphorylates mAtg13, FIP200 and itself to initiate autophagy. Regarding the substrates of the Atg1 kinase during autophagy, it is suggested that mAtg13 and FIP200 are phosphorylated by ULKs, and ULKs also undergo autophosphorylation, which is conducive to a conformational change and autophagy induction. Mammalian target of rapamycin (mTOR) can phosphorylate and inactivate ULKs and Atg13 under nutrient-rich conditions. Upon mTOR inhibition by starvation or rapamycin, ULK1 and ULK2 are activated and phosphorylate Atg13 and FIP200, which are essential for autophagy activity. Recent studies have suggested that Atg13 may be phosphorylated by TOR or Atg1/ULKs on differ-

ent residues [10]. It is likely that phosphorylation of Atg13 is dependent to a greater extent on TOR in yeast, but on Atg1 in Drosophila.

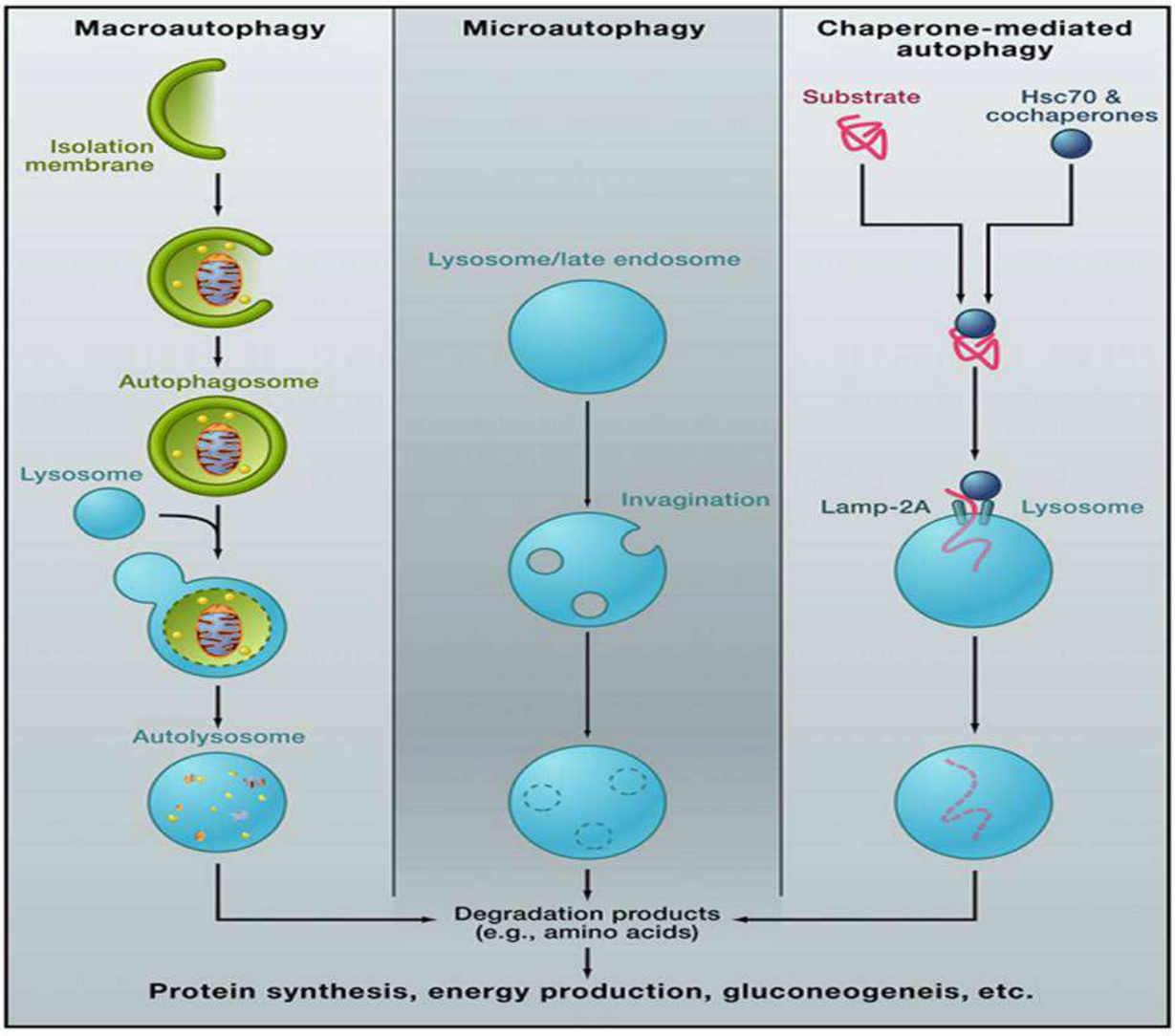

Figure 1. Different Types of Autophagy (Cite from < Mizushima N, Komatsu M. Cell 2011;147(4) 728-741>).

The conjugation of Atg12 and Atg8 is essential for the formation of autophagosomes. Atg12 was the first ubiquitin-like Atg protein to be identified, which can be activated by Atg7 and Atg10. Then it is conjugated to Atg5 and promotes the formation of the autophagy precursor [11]. The amino-acid sequence of Atg12 ends with a glycine residue and there is no protease involved in Atg12 conjugation. Analogous to ubiquitination, there is an E1-like enzyme, Atg7, and Atg12 is activated by forming a thioester bond between the C-terminal Gly 186 of Atg12 and the Cys 507 of Atg7. After activation, Atg12 is transferred to Atg10, which is an E2 enzyme, and is eventually conjugated to the target protein Atg5 at Lys 149 through an isopeptide bond. There is no typical E3 enzyme involved in Atg12-Atg5 conjugation. Atg5 interacts further with a small coiled-coil protein, Atg16, and Atg12-Atg5-Atg16 forms a mul-

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timeric complex through the homo-oligomerization of Atg16. The ubiquitin-like (Ubl) protein Atg8 is attached to phosphatidylethanolamine (PE). The C-terminal Arg 117 residue of Atg8 is initially proteolytically removed by a cysteine protease, Atg4, to expose Gly 116. This exposed glycine forms a thioester bond with Cys 507 of Atg7, which is also the site that participates in the Atg12-Atg5 conjugation. This feature differentiates Atg7 from most other E1 enzymes, which activate single Ubl proteins. Activated Atg8 is then transferred to the E2-like enzyme Atg3, also through a thioester bond. In the final step of Atg8 lipidation, Gly 116 of Atg8 is conjugated to PE through an amide bond; Atg8-PE exists in a tightly membrane-associated form. Microtubule-associated protein l light chain 3 (LC3) is the mammalian homologues of Atg8. LC3 is activated by Atg7, transferred to Atg3, and conjugated to phosphatidylethanolamine (PE) on the surface of autophagic vacuoles membranes to promote the forming of autophagosomes [12].

Recently, the PI3K-Becline 1 complex and Atg9 have been shown to be the essential components involved in autophagy signaling and the membrane transportation of autophagic vacuoles. This autophagy-specific class III-PI3K-Becline 1 complex appears to be essential to recruit the Atg12-Atg5 conjugation to the pre-autophagosomal structure. Atg12-Atg5 conjugation is then required for the elongation of the isolation membrane and for the proper localization of conjugated LC3/Atg8 [13]. Atg9 is required for autophagy in both yeast and mammalian cells and has been speculated to be involved in delivery of membrane lipids to form autophagosomes. In mammalian cells, mAtg9 traffics between the trans-Golgi network, endosomes and newly formed autophagosomes [14].

Cells regulate autophagy through a set of precise signaling pathways including integrating nutriment, growth factors, hormones, stress and intracellular energy information. A key regulator point of autophagy in mammals is kinase mTOR. The kinase TOR is a major evolutionarily conserved sensor in the autophagy signaling pathway in eukaryotes, but it also regulates many other aspects of cell function, including transcription, translation, and cell size and cytoskeletal organization. In mammals, mTOR can be included in two different complexes, mTORC1 and mTORC2. Although these two TOR complexes share common components, they display distinct cellular functions and phosphorylate different downstream substrates. The activity of mTORC1 is regulated via the integration of many signals, including growth factors, insulin, nutrients, energy availability, and cell stressors such as hypoxia, osmotic stress, reactive oxygen species (ROS) and viral infection. mTORC1 is the only known target of the drug rapamycin and is required for signaling to ribosomal S6 kinases (S6K) and eIF4E-binding proteins (4EBP1 and 4EBP2). mTORC1 has recently been shown to consist of four proteins: mTOR, mLST8 (also known as GbL), proline-rich PKB/Akt substrate 40-kDa (PRAS40), and raptor (regulatory associated protein of mTOR); and it plays a major role in controlling translation and cell growth in response to nutrients. The adaptor protein is common to both mTOR complexes. Raptor binds mTOR, S6K and 4EBP1/2 and facilitates mTOR phosphorylation of these molecules; but whether raptor enhances or represses mTOR kinase activity remains unclear [15]. Unlike mTORC1, mTORC2 has some functions that cannot be inhibited by rapamycin, including the control of actin cytoskeleton dynamics. The mTORC2 complex consists of mTOR, mLST8, mammalian stress-

activated protein kinase-interacting protein 1 (mSin1), and rapamycin-insensitive companion of mTOR (rictor). Recent studies indicate that when eutrophy, mTOR activates the PI3K-I/ protein kinase B (PKB) signaling pathway, it leads multiple serine sites to phosphorylation and then reduces the affinity of Atg13 and Atg1. Since Atg1-Atg13 compounds decreased, Atg9 cannot be transferred to the autophagosome formation sites and autophagy was inhibited.

AMP-activated protein kinase (AMPK) was initially identified as a serine/threonine kinase that negatively regulates several key enzymes of the lipid anabolism. Meanwhile, AMPK is regarded as the major energy-sensing kinase that activates a whole variety of catabolic processes in multicellular organisms such as glucose uptake and metabolism, while simultaneously inhibiting several anabolic pathways, such as lipid, protein, and carbohydrate biosynthesis. Activated AMPK can inhibit mTOR by interfering with the activity of GTPase Rheb and with protein synthesis, degrade the phosphorylation of ULK1 and promote disintegration of ULK1 from the mTOR compounds. In starved cells, when the AMP/ATP ratio increases; the binding of AMP to AMPK promotes its activation by the AMPK kinase LKB. Moreover, Ca^{2+}/calmodulin-dependent kinase kinase beta (CaMKK-beta) has been identified as being an AMPK kinase. The activity of AMPK is required for autophagy to be induced in response to starvation in mammalian cells and in yeast in a TORC1-dependent manner. Moreover, autophagy induction is also dependent on the inhibition of mTORC1 by AMPK in non-starved cells in response to an increase in free cytosolic Ca^{2+}. In this setting, the activation of AMPK and stimulation of autophagy are dependent on CaMKK-beta. The induction of autophagy through AMPK activation probably also occurs in other settings, such as hypoxia. AMPK is probably a general regulator of autophagy upstream of mTOR [16]. Another potential candidate of autophagy regulation down-stream of AMPK is elongation factor-2 kinase (eEF-2 kinase), which controls the rate of peptide elongation [17]. Activation of eEF-2 kinase increases autophagy and slows protein translation. The activity of eEF-2 kinase is regulated by mTOR, S6K, and AMPK. During periods of ATP depletion, AMPK is activated and eEF-2 kinase is phosphorylated, leading to a balance between the inhibition of peptide elongation and the induction of autophagy. However, how eEF-2 kinase impinges on the molecular machinery of autophagy remains to be elucidated.

There also is an indirectly-TOR-dependent signaling pathway, named class I PI3K/Akt pathway, which when responding to insulin-like and other growth factor signals, the signaling molecules link receptor tyrosine kinases to activate TOR kinase and thereby repress autophagy. In addition, autophagy can mediate inactive proteins to degrade to amino acids and then provide metabolic substrates for cardiac development and ischemic hypoxia forming a cardiac protection method. Autophagy is among the important mechanisms of hypoxic adaptation and is perhaps one of the last resorts for the salvage of ATP in hypoxic cells and organs.

p53 is a responsive stress protein which also plays a crucial role in the regulation of autophagy through the transcription-dependent/independent pathways. In the transcription-independent pathway, p53 can activate AMPK and down-regulate mTOR; in the transcription-dependent pathway, through the up-regulation of PTEN (inhibitor of mTOR), the tuberous

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sclerosis-1 (TSC1) gene or cell death gene damage-regulated autophagy modulator 1 (DRAM1), mTOR is down-regulated, and autophagy is induced (Figure 2). In addition, c-jun N signal kinases (JNK), GTPases, Erk1/2, ceramide are also involved in regulating autophagy. The cytoplasmic form of p53 has been shown to have an inhibitory effect on autophagy, suggesting that activation of autophagy by p53 depends on its transactivating effect on genes such as DRAM1 [18].

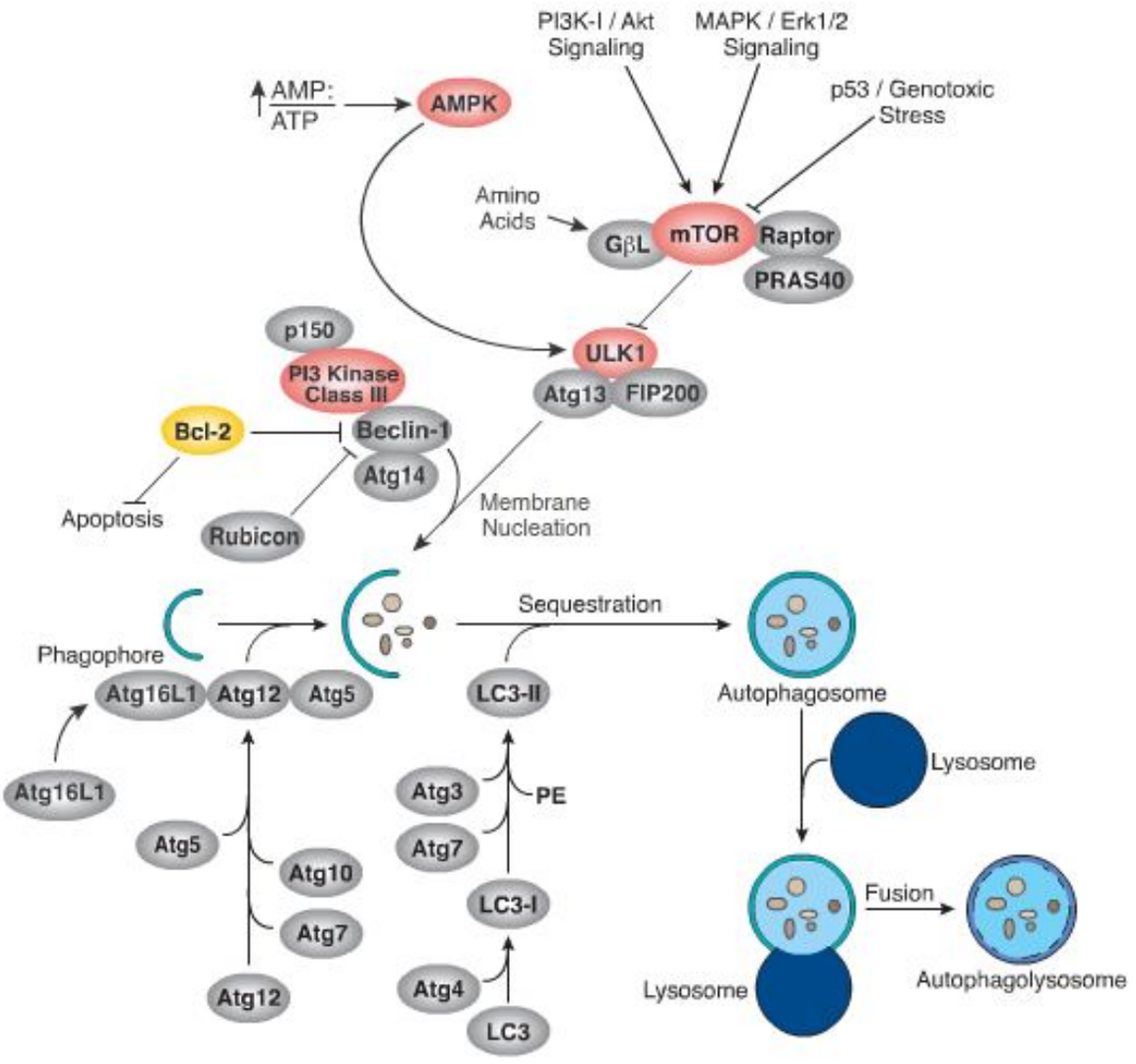

Figure 2. Signaling Pathway of Autophagy (Cite from http://www.cellsignal.com/).

4. The role of autophagy in the maintenance of normal myocardium

In the basal state, autophagy showed low expression in the heart to maintain normal myocardial function. And most of its function is to perform homeostatic functions by eliminating long-live organelles and proteins. Autophagy can be upregulated rapidly when myocardium cells need to generate intracellular nutrients and energy, for example during starvation or trophic factor withdrawal. Nutritional status, hormonal factors, and other cues like temperature, oxygen concentrations, and cell density are important in the control of autophagy.

The molecular mechanism of autophagy is still poorly understood. There are more than 30 genes that have been confirmed to be related to autophagy, and half of them are highly conservative in most metazoa [19]. Knockout Atg5 gene (a decisive gene in autophagy) in early stage adult rat hearts has showed no obvious abnormality, however, after increased afterload for one week, left ventricular can cause myocardial ubiquitination and mitochondrial aggregation, resulting in myocardial hypertrophy and decrease in myocardial contractility, which indicates that autophagy may play an important role in maintenance of homeostasis, size of myocardium, general construction and function of myocardial cells. Paradoxically, partially reduced autophagic activity caused by heterozygous deletion of Beclin 1 improves cardiac function upon pressure overload. Consistent with this phenomenon, partial suppression of autophagy with histone deacetylase (HDAC) inhibitors can ameliorate pressure overload-induced cardiac hypertrophy in mice [20]. These data suggest that partial, but not complete, suppression of autophagy may be beneficial.

In short, autophagy adapts to the myocardial energy demand by maintaining energy metabolism, so as to protect the myocardial function.

5. The protective effect of autophagy in myocardial ischemia/hypoxia

In recent years, a large number of studies suggested that autophagy played a protective role to rescue cardiomyocytes in ischemia/hypoxia. In [21], researchers discovered that 40 mins of hypoxia induced significant autophagosome and autolysosome formation, according to a rabbit hypoxic heart model. At the same time, ultrastructural analysis revealed autophagosomes in close proximity to swollen and fragmented mitochondria. And in rodents, 30 mins after ischemia induced dramatic up-regulation of autophagosome formation [22]. Previous report also demonstrated that the level of autophagy was rapidly increased within 30 mins after coronary ligation in mice, especially in the risk area (salvaged cardiomyocytes bordering the infarcted area) [23]. Recent work has revealed that inactivation of hypoxia-inducible factor 1α (HIF-1α) in fibroblasts blunts hypoxia-induced autophagy [24]. These results strongly suggested that autophagy was activated by myocardial ischemia/hypoxia. Furthermore, these investigators reported that suppression of autophagy using 3-methyladenine or bafilomycin A1 enhanced myocyte death triggered by glucose deprivation. During postinfarction cardiac remodeling, lysosomes and autophagosomes became more numerous in the cardiomyocytes, thus ensuring that autophagy could provide enough energy to cardiomyo-

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cytes. The protective effects of autophagy may be that cells were likely to be provided energy, free amino acids and fatty acids through decomposition of their own material.

Additionally, autophagy may maintain cardiomyocyte survival after ischemia/hypoxia by inhibiting apoptosis [25]. Autophagy is a recycling process of cytoplasmic components, such as long-lived proteins and organelles. The prosurvival role of autophagy has been observed in yeast, plants, flies, and mammals. Inhibition of autophagy results in accumulation of cytoplasmic components and promotion of apoptosis. Treatment with pharmacological autophagy inhibitors and knockdown of Atg genes (Atg5, Atg10, Atg12, and Beclin 1) can increase apoptosis and cell death in nutrient-deprived cells. In reference [26], autophagy delayed apoptotic cell death in breast cancer cells following DNA damage. These results suggested that the effect of inhibiting apoptosis may be related to autophagosomes wrapping damaged mitochondria, because it will not only prevent the release of cytochrome C, but also inhibit the formation of apoptotic bodies.

Numerous studies show that mTOR is a key factor in regulating autophagy in ischemia/hypoxia. In mammalian cells, phosphorylation of mTOR inhibits cell autophagy. Conversely, dephosphorylation of mTOR enhances autophagy. The activity of mTOR is adjusted by many factors. AMPK is the important factor which would inhibit the activity of mTOR to enhance autophagy in ischemia. AMPK serves as a general integrator of metabolic responses to changes in energy availability and is activated in response to elevations of the AMP/ATP ratio. Data in reference [27] suggested that autophagy has been reported to be up-regulated in response to reduced cellular content of ATP. In cultured cardiac myocytes, glucose deprivation caused significant reduction in the levels of ATP, which coincided with up-regulation of autophagy. Moreover, myocardial ischemia causes a decrease in ATP levels and an increase in the AMP/ATP ratio, resulting in activation of the AMPK [28]. Under conditions of stress (including hypoxia and ischemia), a signaling cascade is initiated involving phosphorylation of AMPK and subsequent inhibition of mTOR. Inhibition of mTOR, in concert with other protein partners, provides the critical step in initiating autophagosome formation.

6. The expression and regulation of autophagy during myocardial ischemia/reperfusion

Autophagy is induced during myocardial ischemia. Although it was speculated that activation of autophagy may be reverted when ischemia is relieved, in fact, autophagy may be further enhanced by reperfusion [22, 29]. A variety of factors that can regulate the autophagy during myocardial ischemia/reperfusion, such as ROS generated by mitochondrial respiration, endoplasmic reticulum stress, calcium, vitamin D compounds, ATP, thapsigargin, calcium protease, and so on. Different signal transduction pathways are involved in the occurrence of autophagy at different stages of myocardial ischemia/reperfusion.

6.1. The role of Beclin 1 in the occurrence of autophagy during myocardial ischemia/reperfusion

Induction of autophagy in the ischemic phase was accompanied by activation of AMPK and mTOR. In contrast, autophagy during reperfusion was accompanied by upregulation of Beclin 1 rather than by activation of AMPK. Induction of autophagy and cardiac injury during the reperfusion phase was significantly attenuated in Beclin 1+/- mice. Collectively, in the ischemic heart, autophagy is stimulated through an AMPK-dependent mechanism, whereas ischemia/reperfusion stimulates autophagy through a Beclin 1-dependent but not an AMPK-independent pathway [22]. Using cultured cardiomyocytes, previous studies have demonstrated that the inhibition of autophagy by urocortin during the reperfusion phase is mediated in part by inhibition of Beclin 1 expression, an effect which is mediated by activation of the PI3K/Akt pathway [30]. Recent experimental data also show that the clearance of autophagosomes is impaired in myocardial reperfusion injury which is mediated in part by ROS-induced decline in LAMP-2A and upregulation of Beclin 1, contributing to increased cardiomyocyte death [31, 32]. Thus, ROS and ROS-mediated upregulation of Beclin 1 in the myocardial reperfusion phase may play an important role in the occurrence of autophagy. Therefore, it is now widely recognized that the autophagy was induced through the AMPK-eEF2K/mTOR pathway during the ischemic phase of ischemic/reperfusion injury, but was triggered through the Class III PI3K/Beclin 1 pathway during the reperfusion phase.

6.2. The role of Bcl-2 family members in the occurrence of autophagy during myocardial ischemia/reperfusion

It is well known that the Bcl-2 family proteins play essential roles in regulating apoptosis in the cardiovascular system, and several studies have revealed that the Bcl-2 family members (Bnip3, Bcl-2, Bcl-XL, Bax, etc.) also play important roles in the induction of autophagy during myocardial ischemia/reperfusion injury.

6.2.1. Bnip3

Bnip3 (Bcl-2/adenovirus E1B-19 KD interacting protein 3) with a single Bcl-2 homology 3 (BH3) domain is a pro-apoptotic Bcl-2 family protein which is most sensitive to hypoxia and plays an important role in myocardial ischemia/reperfusion injury. It was found that the overexpression of Bnip3 significantly increased autophagy, whereas, overexpression of the dominant-negative Bnip3 significantly reduced autophagy induced by myocardial ischemia/reperfusion in HL-1 cardiac myocytes [33]. These results suggest that Bnip3 plays a fundamental role in the induction of autophagy during myocardial ischemia and reperfusion. However, more studies are still needed to clarify the role of Bnip3 in response to ischemia/reperfusion, and the most reasonable explanation is that the mitochondrial dysfunction caused by Bnip3 can enhance the level of autophagy in order to remove damaged organelles.

6.2.2. Bcl-2/Bcl-XL and Bax

Bcl-2 family members are key modulators of apoptosis that have recently been shown to also regulate autophagy. Transgenic mice overexpressing the anti-apoptotic human Bcl-2 cDNA in the heart is effective at reducing myocardial reperfusion injury and improving heart function [34, 35]. However, blockage of the activity of the proapoptotic molecule Bax in a knockout mouse model attenuates ischemia/reperfusion injury [36]. Recent reports demonstrated that Beclin 1 possessed a BH3 domain. The BH3 domain of Beclin 1 is bound to and inhibited by Bcl-2 or Bcl-XL [37, 38]. A BH3 mutant of Beclin1 which has reduced affinity for Bcl-XL/Bcl-2 was a more potent inducer of autophagy than wild type Beclin 1. Overexpression of Bcl-2 in the heart reduced starvation-induced autophagy. Thus, Bcl-2 not only functions as an antiapoptotic protein, but also as an antiautophagy protein via its inhibitory interaction with Beclin 1. These antiapoptosis and antiautophagy functions of Bcl-2 may protect the myocardium against ischemia/reperfusion injury [39]. Meanwhile, some studies found that Bnip3 enhanced autophagy, possibly due to competitive disruption of Bcl-2 binding to Beclin 1 [24] or interacting with Rheb to inhibit mTOR [40]. Of course, more studies are needed to clarify these relationships.

6.3. The role of angiotensin II (Ang II) receptor signaling in the induction of autophagy during myocardial ischemia/reperfusion

Recent report demonstrated that overexpression of Ang II type 1 (AT1) receptor caused a significant increase in autophagy after treatment with Ang II in cultured neonatal rat cardiomyocytes. However, overexpression of the Ang II type 2 (AT2) receptor can inhibit autophagy in an Ang II-independent manner. Neonatal cardiomyocytes cultured from hypertrophic heart rats (HHRs) were more susceptible to AT1 receptor-stimulated autophagy than cardiomyocytes from normal heart rats (NHRs). Moreover, there was a greater up-regulation of autophagic markers in adult HHR hearts than in NHR hearts following ischemia/reperfusion in vitro [41]. Additionally, AT1 receptor blockaded with olmesartan plays a protective role in myocardial ischemia-reperfusion injury [42]. Therefore, it is inferred that Ang II/AT1 receptor signaling might also be involved in the stimulation of autophagy during myocardial ischemia/reperfusion.

Thus, in addition to the Beclin 1, many Bcl-2 family members and angiotensin II/AT1 receptor signaling may also be involved in the stimulation of autophagy, but additional studies are needed to clarify the role of these pathways in the occurrence of autophagy during ischemia/reperfusion injury of the heart.

7. The role of autophagy in myocardial ischemia/reperfusion

The autophagy is induced during the ischemia/reperfusion process; however, the role of autophagy during myocardial ischemia/reperfusion is still inconclusive.

It is well-documented that autophagy occurs at basal levels but can be further induced by stresses, such as nutrient depletion. Autolysosomal degradation of membrane lipids and

proteins generate free fatty acids and amino acids, which can be reused to maintain mitochondrial ATP production and protein synthesis and promote cell survival. When the myocardium was suppressed with ischemia, the blood supply was decreased and the energy was insufficient, which means that there was an inadequate supply of nutrients. In these conditions, AMPK acts as a sensor for energy deprivation and activation of AMPK mediates metabolic adaptation during ischemia.

Many studies have shown that the autophagy induced by lack of blood supply plays a protective effect during periods of ischemia. For example, see [43], autophagy is significantly up-regulated during chronic ischemia in the pig heart. In this model, the level of autophagy was inversely correlated with that of apoptosis in the ischemic area. The ischemic area was recovered when the coronary flow was restored, suggesting that autophagy may protect myocardium from apoptosis during hibernation. Inhibition of endogenous AMPK suppressed autophagy during prolonged ischemia, which was accompanied by enlargement of the myocardial infarction. Although one may speculate that activation of autophagy may be reverted as soon as ischemia is relieved, the level of autophagy in fact further increases during reperfusion. However, a higher level of autophagy is not due to the lack of energy during blood flow restoration after reperfusion. Mechanisms mediating autophagy during reperfusion appear different from those involved in autophagy during ischemia. Energy crisis, a major stimulus for autophagy, in the heart is at least partially resolved at the time of reperfusion [32]. Instead, ROS appears to be a major promoter of autophagy during reperfusion. ROS induces mitochondrial damage, as evidenced by mitochondrial permeability transition pore (mPTP) opening and mitochondrial fragmentation, which in turn promotes autophagy and/or mitophagy, a specialized form of autophagy which removes mitochondria [44]. ROS oxidizes and inhibits the cysteine protease activity of Atg4, which results in LC3 lipidation and autophagy.

Whether autophagy induced during reperfusion is beneficial or detrimental remains controversial. Previous data [32] have shown that, although autophagic flux is inhibited during ischemia/reperfusion, enhancing autophagic flux during ischemia/reperfusion protects against ischemia/reperfusion injury in cardiomyocytes in vitro. The number of Bax (+) cardiac cells induced by reperfusion was significantly increased when Beclin-1 or Atg-5 were knocked out, but was reduced when there was an overexpression of Beclin-1 [45]. In an in vivo model of myocardial ischemia/reperfusion in pigs, autophagy was significantly activated when the coronary perfusion is restored, which was accompanied by reduction of apoptosis in myocardial cells and almost complete recovery of cardiac function [46]. Other experiments have also demonstrated that autophagy activation during myocardial ischemia/reperfusion can remove the damaged mitochondria caused by Bnip3. These results indicate that autophagy may promote cell survival during myocardial ischemia/reperfusion injury.

In contrast, study in reference [30] showed that inhibiting autophagy by treatment with 3-methyladenine or by Beclin1 knock down increases the survival of cardiomyocytes after ischemia/reperfusion in vitro. As results in [22], the myocardial infarct size increased by 40% in rats subjected to reperfusion, while the infarct size was down-regulated to

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20% after the autophagy gene Beclin1 knockout. Inhibition of cathepsin, which can degrade autophagosomes, can significantly reduce myocardial cell damage and apoptosis during the reperfusion phase. Therefore, the roles of autophagy are not very clear during myocardial reperfusion. Further studies are needed to investigate the real roles of autophagy in myocardial ischemia/reperfusion injury. However, it is still speculated that the excessive degradation of important proteins and organelles by autophagy will cause cell death.

8. The role of autophagy in aging hearts subjected to ischemia/reperfusion

Aging is characterized by a progressive accumulation of damaged cells and organs. Autophagy degraded damaged organelles and macromolecule materials in stress, which prolonged life span [47]. Autophagy, including the autophagosome formation, the maturation, and the efficiency of autophagosome-lysosome fusion, as well as the proteolysis activity of lysosomes, declines with age [48]. All of these factors induce an abundance of the lipofuscin polymer accumulated in lysosomes. The lipofuscin polymer could not be degraded by lysosomes. The lysosome with an abundance of lipofuscin polymer lost its bio-function. Therefore, some damaged organelles and macromolecule materials could not be cleaned up, which accounted for the aging process. Several studies supported enhancing autophagy as the most efficient anti-aging intervention [49, 50].

In the heart, autophagy maintains a low basal level to perform biological functions, such as degrading dysfunctional organelles, maintaining cardiac morphology and function [51, 52]. However, autophagy in cardiomyocytes were up-regulated in response to environmental stress conditions, such as ATP depletion (e.g. during starvation), oxidative damage, and mitochondrial permeability transition pore opening (e.g. myocardial ischemia/reperfusion injury) [53, 54]. Activation of autophagy during ischemia is essential for cell survival and maintenance of cardiac function. However, autophagy was activated in the heart subjected to ischemia/reperfusion. Recent reviews show autophagy during reperfusion could be either protective or detrimental [41]. Serious induction of autophagy accompanied by robust up-regulation of Beclin-1 could cause autophagic cell death, thereby proving to be detrimental. If ischemia is mild, activation of autophagy during reperfusion may be modest and thus may not be harmful.

Until now, there is little information related to autophagy in myocardial ischemia/reperfusion injury in aging. Our primary data showed that ischemia/reperfusion injury was more serious in aging hearts compared with young hearts. Beclin-1 was increased in aging hearts subjected to ischemia/reperfusion, which indicated activated autophagy. However, further research is needed to know the exact role of autophagy in myocardial ischemia/reperfusion injury, especially in aging. Moreover, recent studies suggest that autophagy is one of the important mechanisms in myocardial ischemia/reperfusion preconditioning. Decreased autophagy may contribute to the weakened protective role of preconditioning in aging hearts

subjected to ischemia/reperfusion. Therefore, excessive autophagy results in autophagic cell death and loss of cardiomyocytes, responsible for the worsening of aging myocardial ischemia/reperfusion injury. The molecular transduction pathways need to be investigated further. It could help to develop therapies that up-regulate the repair qualities of the autophagic process and down-regulate the cell death aspects, which would be of great value in the treatment of aging myocardial ischemia/reperfusion injury.

9. The investigative methods of autophagy

9.1. Electron microscopy

Electron microscopy is the most reliable method for testing autophagy at present and can be used to quantify the autophagic activity of cells. In electron microscopy, the autophagosome is composed of double membrane structures that wrap abnormal cytoplasmic material. Then, the autolysosome is composed of the monolayer membrane structure which wraps cytoplasm ingredients at different degradation stages. Because of the difficulty of distinguishing between autophagosomes and autolysosomes, both of them are often called 'autophagic vacuoles'. The proportion of autophagic vacuoles is calcultaed accounting for the total area or volume of cytoplasm through electron microscopy, which can be used to quantify cell autophagic activity.

9.2. Specific markers

Atg8 includes three kinds of homology in humans: GABARAP, GATE-16 and LC3. The C-terminal proteolysis of LC3 is processed by Atg4, based on the residues Phe80 and Leu82 of LC3 that may be recognized by Atg4, and immediately follows synthesis to yield a soluble form, LC3-I. LC3-I is converted to a membrane bound form, LC3-II, through a ubiquitin-like reaction involving Atg7, a ubiquitin-acivating enzyme (E1)-like enzyme, and Atg3, a ubiquitin-conjugating enzyme (E1)-like enzyme (E2). LC3-II combines to phosphatidyl ethanolamine (PE) on the membrane surface of autophagic vacuoles. In addition, LC3-II is easily located within the cell after the formation of the fusion protein with the green fluorescent protein (GFP). Therefore, GFP-LC3 II is usually used as the marker protein of the autophagosome membrane in mammalian cells.

Beclin 1 is involved in the formation of autophagosomes, which are the mammalian yeast homologues of Apg6/Vps30. Some researches show that autophagy is significantly attenuated in Beclin 1+/- mice, but apoptosis is normal. These results indicate that Beclin 1 is a significant positive control gene in autophagy. Therefore, by testing the expression level of Beclin1, combined with other biochemistry indexes, the autophagic activity of cells can be monitored and judged dynamically.

9.3. Monodansylcadaverine (MDC) dyeing

MDC is a kind of fluorescent dye, which is used as a tracer of the autophagic vacuoles. MDC can specifically bind to the ubiquitin-proteasome sample protein binding systems (Atg8). In addition, MDC can be absorbed by cells and selectively gathered in autophagic vesicles, showing punctuate structure under fluorescence microscope. Therefore, the quantitative detection of autophagy uses this method. However, most of the MDC is not marked in GFP-LC3. However, MDC is not a reliable marker of autophagosomes. Therefore, other experimental evidences that represent the autophagic activity of cells are needed.

9.4. Specific agonists and inhibitors

Rapamycin is a novel macrolide immunosuppressant, and induces cell autophagy by inhibiting the mTOR pathway. In scientific studies, Rapamycin is the specific agonist of autophagy. The main inhibitors of the phosphor-lipin acid radical inositol 3 kinase include 3-MA, which can specifically block the fusion of autophagic vacuoles and lysosomes. Rapamycin is widely used as an inhibitor of autophagy, and Wortmannin, LY29400297 and Bafilomycin A1 are included.

10. Perspectives

Autophagy is intimately involved not only in the physiology of the heart, but also in development of the ischemic heart. The regulation of autophagy may be a new approach for the treatment of ischemic heart disease. However, to translate the knowledge of autophagy into treatment of ischemic heart disease, it is necessary to know more precisely about the formation, function, mechanism, and regulation of autophagy. When autophagy is protective and when it is detrimental for the ischemic heart needs further clarification. Another problem is how to regulate autophagy without affecting other life activities, since even the evolutionarily conserved autophagy genes also have autophagy-dependent functions. There is continuing research on this topic. In addition, the signaling mechanisms positively or negatively regulating autophagy in the heart have not been completely elucidated. Judging from the diversity of autophagy regulators, it is believed that more unknown signal transduction pathways will also be proven to be involved in the activation of autophagy. In short, only clarifying the activation mechanism, function, time course, and its relationship with cell death, etc. autophagy can truly benefit patients with ischemic heart disease.

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