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Review · **Open Access** ·

The role and modulation of autophagy in experimental models of myocardial ischemia-reperfusion injury

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Abstract

A physiological sequence called autophagy qualitatively determines cellular viability by removing protein aggregates and damaged cytoplasmic constituents, and contributes significantly to the degree of myocardial ischemia-reperfusion (I/R) injury. This tightly orchestrated catabolic cellular 'housekeeping' process provides cells with a new source of energy to adapt to stressful conditions. This process was first described as a pro-survival mechanism, but increasing evidence suggests that it can also lead to the demise of the cell. Autophagy has been implicated in the pathogenesis of multiple cardiac conditions including myocardial I/R injury. However, a debate persists as to whether autophagy acts as a protective mechanism or contributes to the injurious effects of I/R injury in the heart. This controversy may stem from several factors including the variability in the experimental models and species, and the methodology used to assess autophagy. This review provides updated knowledge on the modulation and role of autophagy in isolated cardiac cells subjected to I/R, and the growing interest towards manipulating autophagy to increase the survival of cardiac myocytes under conditions of stress-most notably being I/R injury. Perturbation of this evolutionarily conserved intracellular cleansing autophagy mechanism, by targeted modulation through, among others, mammalian target of rapamycin (mTOR) inhibitors, adenosine monophosphate-activated protein kinase (AMPK) modulators, calcium lowering agents, resveratrol, longevinex, sirtuin activators, the proapoptotic gene *Bnip3*, IP3 and lysosome inhibitors, may confer resistance to heart cells against I/R induced cell death. Thus, therapeutic manipulation of autophagy in the challenged myocardium may benefit post-infarction cardiac healing and remodeling.

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1 Introduction

Acute myocardial infarction (AMI) results from a disruption of coronary blood flow to the myocardial region that it supplies. With an incidence of 935 per 100,000 persons and resulting in 141,462 deaths in 2006, AMI remains the leading cause of mortality in the United States. In addition, AMI is associated with a heavy financial burden and the American Heart Association has estimated the cost of treating coronary heart disease-related injuries as $165.4 billion for 2009 alone.[1] A prolonged ischemic event triggers several molecular and structural changes which cause damage or death of end stage myocytes thus impairing myocardial function. Currently, the treatment of AMI relies on reperfusion therapy based on inducing pharmacological (e.g., thrombolytics) and/or invasive (e.g., angioplasty) reopening of the occluded coronary artery. However, for the reperfusion therapy to be effective, it has to be initiated as soon as possible after the onset of ischemic insult.[2] If there occurs a delay in restoration of coronary flow, cardiac cell death and ventricular remodeling occurring after the ischemic injury, progress to heart failure in up to 40% of patients.[3] Paradoxically, the reintroduction of oxygen-rich blood to the ischemic tissue also has detrimental effects, collectively termed as "reperfusion injury". Thus, the prevention of

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post-ischemic cardiac cell death may represent the best therapeutic modality to limit damage incurred by the ischemic myocardium.

During the past decades, extensive investigation has led to the development of cardioprotective strategies against ischemia-reperfusion (I/R) injury. The description of ischemic preconditioning,[4] where multiple brief periods of ischemia protect the myocardium from a subsequent sustained ischemic insult, had a major impact in the field of cardioprotection. This was followed by the discovery of the phenomenon of ischemic post-conditioning, where repetitive bouts of ischemia applied at the onset of reperfusion, would also protect the heart from myocardial I/R injury.[5] Indeed, it has also been reported that repeated ischemia at sites remote from the heart can also be cardioprotective. Considerable advances have been made in recognizing the molecular mechanisms that determine the protection afforded by these strategies. This has opened new avenues for the identification of novel therapeutic agents that mimic the cardioprotective effects of ischemic pre- and post-conditioning (pharmacological pre- and post-conditioning). In addition, pharmacological agents targeting key signaling effectors involved in the ischemic and reperfusion cascades have also been developed.[6] While the majority of these therapeutic strategies have proven to be efficient in protecting the heart against I/R injury in animal models, most of them have yielded disappointing results in clinical trials.[7] Despite these setbacks, new potential "druggable" targets for the clinical management of myocardial I/R injury keep emerging from the continued progress in our understanding of the pathogenesis of I/R injury.

There is some evidence that autophagy plays a role, at least in preconditioning. For example, inhibition of autophagy with a dominant negative inhibitor of Atg5, a critical component of the autophagy cascade, abolishes preconditioning in HL-1 cells.[8] It has also been shown that preconditioning requires selective Parkin-dependent mitophagy,[9] suggesting that the preconditioning effect depends on removal of damaged or poorly functioning mitochondria.

At the clinical level, myocardial injury is characterized by myocardial stunning, arrhythmias, microvascular dysfunction and myocardial cell death.[10] Myocardial cell death is governed by the complex biochemical cascades set in motion by both ischemia and reperfusion, (Figure 1).[11] Broadly, myocyte injury can progress along two distinct cell death pathways-necrosis and apoptosis.[12] Necrosis develops as an acute response to lethal exogenous stimuli under pathological conditions. Cell death via necrosis is defined by cellular swelling, rupture of the cell membrane with consequent release of cellular components (e.g., creatine kinase, troponin), and subsequent inflammation. Apoptosis is an adenosine triphosphate (ATP)-dependent process that progresses through a well-orchestrated series of molecular, biochemical, and morphological events that do not result in cell rupture and which do not provoke an inflammatory response.

Apoptotic cells are normally phagocytosed by surrounding cells; but if this process is delayed, apoptotic cells undergo secondary necrosis. Accordingly, AMI which results in a higher proportion of (either primary or secondary) necrotic myocytes will result in a larger lesion than one in which apoptosis is predominant due to the associated inflammatory response. The relative contribution of necrosis and apoptosis to the total cardiac cell loss following both ischemia and reperfusion is not well established. The degree of ATP depletion appears to be a determining factor in the mode of death that injured myocytes would undergo.[13] In the infarct core, which is completely deprived of oxygen and energy, myocytes die by necrosis. In contrast, in the peri-infarct penumbra, which is hypoxic, myocardial cell death occurs via both apoptosis and necrosis.[14] Necrosis represents the predominant mechanism of myocyte death during ischemia. However, necrosis has also been observed during the reperfusion phase. The presence of necrotic cells was detected in the infarct early following the restoration of blood flow but also 24 h post-reperfusion. Also, apoptosis has been reported to occur in the majority of the injured rat cardiomyocytes during the first hours after coronary occlusion (i.e., ischemia).[15] Conversely, Gottlieb, *et al*.[16] have observed the presence of apoptotic cells in reperfused but not in ischemic rabbit hearts. Interestingly, evidence has also pointed to a stepwise progression of the apoptotic program where apoptosis could be initiated during ischemia and completed during reperfusion.[17–19] The discrepancy in these findings may be due to the wide variety of experimental models. Each model has different technical limitations in its ability to estimate the incidence and timing of necrosis and apoptosis.[20]

The picture of myocardial cell death was further complicated by the observation that autophagy is involved in the pathogenesis of myocardial I/R injury. Autophagy accompanies many pathophysiological processes. A plethora of information is available about the activation of autophagy under conditions such as nutrient depletion, presence of reactive oxygen species (ROS), mitochondrial transition pore opening and I/R. Autophagy, together with apoptosis and necrosis, plays a dichotomous 'survival and death' role in cell homeostasis. Since it could be exploited therapeutically, it is important to study the survival role of autophagy during I/R injury in experimental models.

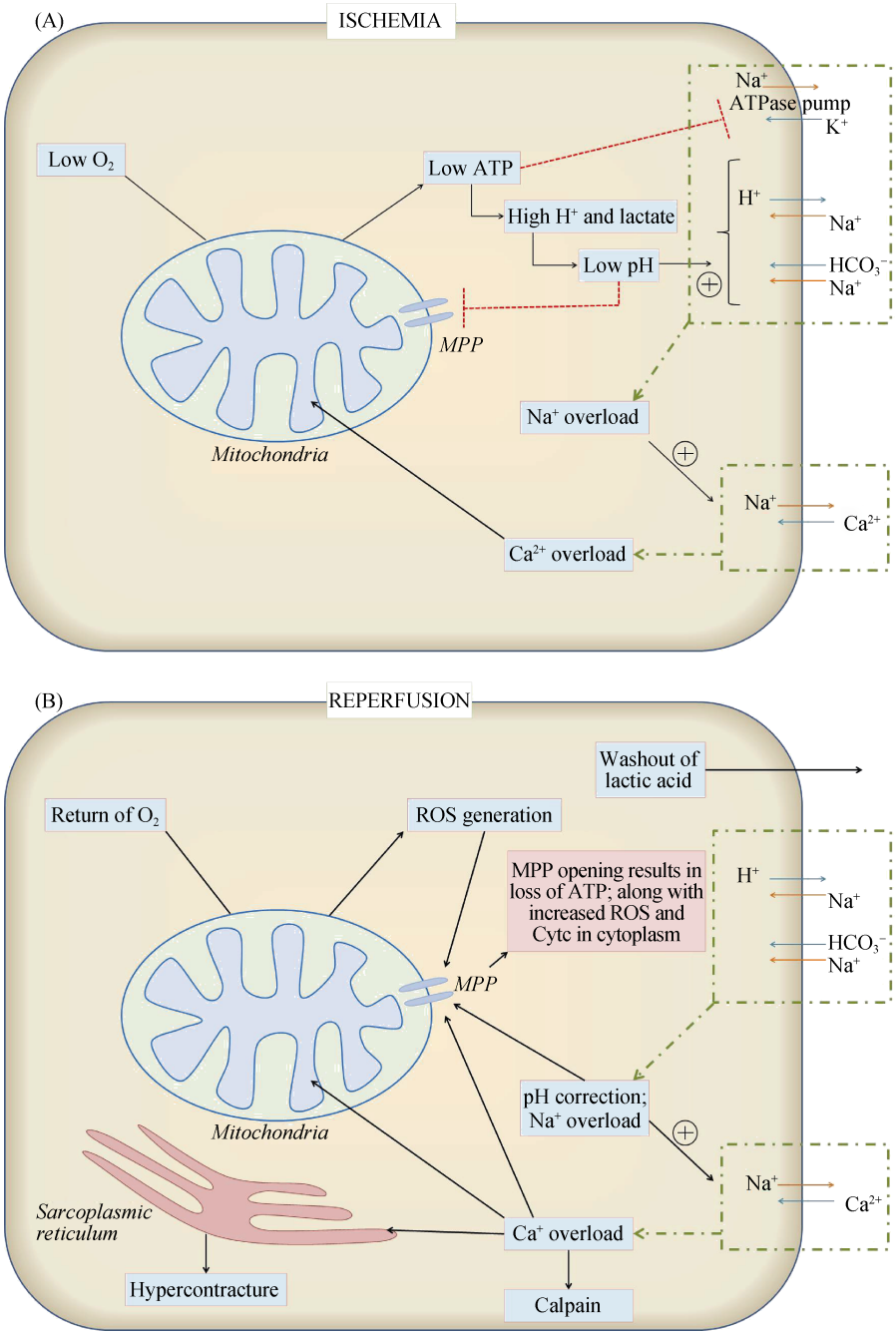

Figure 1. Cellular mechanisms of myocardial I/R injury. (A): The sequence of events elicited by ischemia include: ATP depletion, production of lactic acid, H^+ and CO_2 (anaerobic glycolysis) and acidification, overload of Na^+ (Na^+/H^+ exchanger and inhibition of the Na^+/K^+ ATPase pump), build-up of Ca^{2+} in the cytosol and mitochondria (reversal of Na^+/Ca^{2+} exchanger); (B): Reperfusion promotes the production of ROS, restoration of a physiological pH (wash out of lactic acid, H^+ and activation of Na^+/H^+ exchanger and Na^+/HCO_3^- transporter), Ca^{2+} overload in cytosol, mitochondria and sarcoplasmic reticulum (reversal of Na^+/Ca^{2+} exchanger), restoration of ATP production and opening of the MPP (oxidative stress, normal pH, Ca^{2+} overload). The morphological changes induced by I/R include: (Ⅰ) cytoskeleton fragility, caused by the degradation of α-fodrin mediated by Ca^{2+}-activated calpain, (Ⅱ) hypercontracture, which is caused by the ATP/cytosolic Ca^{2+} overload combination and can spread to adjacent cells, and (Ⅲ) rigor-contracture, which occur when the ATP level is low (a slow ATP production recovery or damaged mitochondria). In addition, the opening of the MPP leads to loss of ATP, damage to the mitochondria, release of ROS and cytochrome C in the cytoplasm. Collectively, these events result in the physical disruption of the sarcolemma and cell death. I/R: ischemia-reperfusion; ROS: reactive oxygen species; MPP: mitochondria permeability pore.

2 The process of autophagy, its regulation and importance in the heart

Macroautophagy (hereafter called autophagy) is an evolutionarily conserved catabolic process that targets dysfunctional or damaged cytoplasmic constituents to the lysosome for degradation and recycling. For example, damaged or dysfunctional mitochondria are physiologically degraded by an autophagic process called mitophagy. Moreover, autophagy is essential for the survival of newborn mice between birth and the onset of suckling.[21] Autophagy occurs constitutively in all eukaryotic cells where it operates as a metabolic homeostatic mechanism.[22] Autophagy can be further activated in response to various physiological and pathological stimuli to either promote cell survival (e.g., during starvation, oxidative stress);[23] or to act as a mode of cell death in its own right (e.g., during development).[24] The evolutionary conservation of the autophagic machinery is revealed through homologous autophagy genes in yeasts, drosophila, vertebrates and man. This library of up to 27 homologous genes and their analogues are known as autophagy-related genes (Atg) following Klionsky in 2003.[25]

Upon induction, autophagy starts with the appearance of a small isolation membrane, the phagophore, which engulfs parts of the cytoplasm and elongates to form a double-membrane vesicle called the autophagosome. The latter undergoes a maturation process and ultimately fuses with a lysosome to form the autophagic vacuole (or autolysosome). The vesicular content is degraded by lysosomal hydrolases, thereby providing the cell with a new source of energy. Autophagy progresses through several steps orchestrated by the Atg genes and proteins localized on the membrane of autophagosome, which includes microtubule-associated protein 1 light chain 3 (LC3) as shown in Figure 2 and Figure 3.

The formation of the autophagosome is initiated by the class III phosphoinositide 3-kinase (PI3-K) protein complex that includes Beclin 1 (Atg6) and involves two ubiquitin-like conjugation systems which result in the formation of *Atg5-Atg12* and LC3.[26] During this process the soluble form of LC3 known as LC3-I is converted into the autophagic vesicle-associated form represented as LC3-II (microtubule-associated protein 1 light chain 3 (LC3)-phosphatidylethanolamine). While the molecular mechanisms involved in each step of the autophagic process are well understood, a debate persists in the field over the origin of the phagophore.

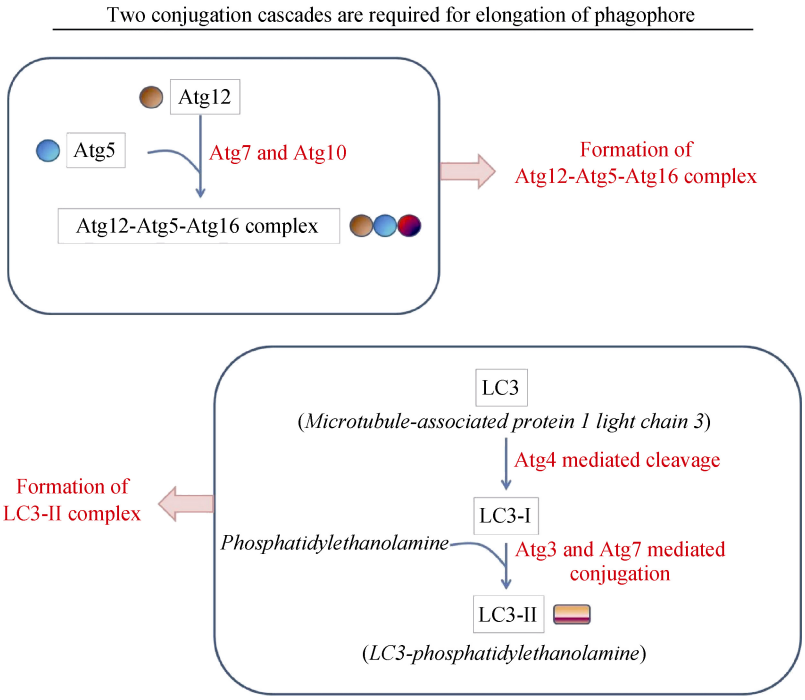

Figure 2. Conjugation cascades involved in elongation of phagophore. The autophagy machinery consists of two conjugation systems required for the elongation and extension of the phagophore: (Ⅰ) Atg5-Atg12, which subsequently oligomerizes with Atg16; and (Ⅱ) LC3-phosphatidylethanolamine, LC3-II. LC3-II is formed as a result of the Atg4-mediated cleavage of cytosolic LC3. The resulting form of LC3, LC3-I is subsequently conjugated to a single PE molecule to form LC3-II, a reaction mediated by Atg3 and Atg7. Atg: autophagy-related genes; LC3: microtubule-associated protein 1 light chain 3; PE: phosphatidylethanolamine.

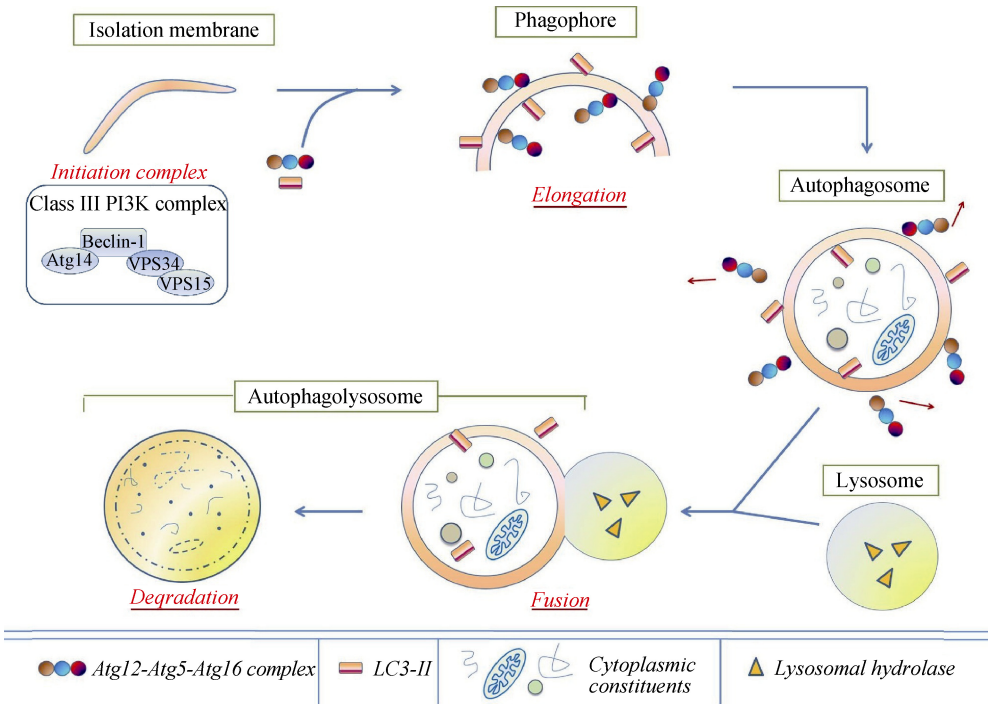

Figure 3. The process of autophagy. Autophagy is initiated with the formation of the phagophore, mediated by the class III PI3-K complex that includes Vps34, Vps15, Atg14 and beclin 1; and progresses through a succession of steps: (Ⅰ) elongation of the phagophore and engulfment of cytoplasmic material targeted for degradation, (Ⅱ) formation of the autophagosome, with delipidation of LC3-II by Atg4, (Ⅲ) fusion of the autophagosome with the lysosome to form the autolysosome, (Ⅳ) degradation of the vesicle content by lysosomal hydrolases and (Ⅴ) recycling of the degradation products (amino acids, lipids and sugars) for ATP production. LC3: microtubule-associated protein 1 light chain 3; PI3-K: class III phosphoinositide 3-kinase.

The endoplasmic reticulum (ER), the trans golgi network (TGN) and the mitochondria have all been proposed as possible sources of autophagosomal membranes.[27] A recent study has provided strong evidence that in normal rat kidney cells, the autophagosomal membranes originate from the outer membrane of mitochondria during starvation but not under ER-stress conditions.[28] Whether this phenomenon can be observed in other cell types and under other stress conditions such as ischemia, awaits further study. Nonetheless, this study has unraveled a potential new interplay between autophagy and the mitochondrion.

Autophagy has also been implicated in post-infarction sequelae. For example, Atg5-deficient mice spontaneously develop left ventricular hypertrophy from the age of about 6 months.[29] Moreover, knockdown of Atg5 results in reduced regression of angiotensin II-induced cardiac hypertrophy,[30] together suggesting that active autophagy may also reduce post-infarct hypertrophy.

2.1 Control of autophagy through multiple signaling pathways

Autophagy is under the control of multiple signaling pathways, including nutrients, stress, hormones, growth factors, and intracellular energy information.[31] The kinase mammalian target of rapamycin (mTOR) is a key modulator of autophagy. mTOR is a sensor of nutrients and is repressed under conditions of nutrient deprivation and hypoxia. Repression of mTOR promotes increased autophagic activity. mTOR receives multiple inputs from metabolic processes and growth factors. Under conditions of growth, mTOR activity is enhanced by factors that activate the class I PI3-K/Akt pathway, which inhibits the tuberous sclerosis complex 1/2 (TSC1/2) and finally increases the activity of Rheb, a guanosine 5'-triphosphatase (GTPase) required for mTOR activity. The stimulation of this cascade results in the inhibition of autophagy. The mTOR complex has two distinct components in mammals, mTORC1 and mTORC2, that phosphorylate different substrates. The mTORC1 structural backbone consists of four different proteins: mTOR itself, mLST8 (GbL), proline-rich PKB/Akt substrate (PRAS40), and raptor protein. This C1 complex plays a major role in signaling to ribosomal S6 kinases (S6K) and eIF4E-binding proteins (4EBP1 and 4EBP2) to regulate cellular growth and translation. The mTORC2 complex is a combination of mTOR, mammalian stress-activated protein kinase-interacting protein 1 (mSin1), mLST8, and rapamy-

cin-insensitive companion of mTOR (ricTOR). Few functions of mTORC2, such as the control of actin cytoskeleton, cannot be inhibited by rapamycin; while all functions of mTORC1 can be inhibited by it.

When nutrient availability is high, mTOR is active resulting in the phosphorylation of multiple serine pathways, and leading to decreased affinity of Atg13-Atg1 components, reduced transfer of *Atg9* to the autophagosome initiation sites and finally inhibition of autophagy. mTOR is also regulated by the adenosine monophosphate-activated protein kinase (AMPK), a sensor of the intracellular AMP/ATP ratio. AMPK is activated in response to an elevated intracellular content of AMP caused by ATP hydrolysis. AMPK suppresses mTOR activity by interfering with GTPase activity, leading to activation of autophagy.

An AMPK kinase, Ca^{2+}/calmodulin-dependent kinase kinase beta (CaMKK-β), stimulates autophagy in the presence of free cytosolic Ca^{2+} in non-starved cells; while the mTORC1 inhibition by AMPK activation induces autophagy in starved and hypoxic cells. AMPK is likely a general regulator of autophagy upstream of mTOR.[32] Recently, activated protein C (APC) has emerged as a novel cardioprotective agent against I/R insult by augmenting AMPK signaling and favorably oxidizing glucose rather than fatty acids as energy substrates in ischemic cardiomyocytes.[33] Elongation factor-2 kinase (eEF-2 kinase), which itself is under the control of mTOR, S6K, and AMPK, elevates autophagic responses and lowers the protein translation rate. In conditions when ATP is depleted, AMPK is activated and eEF-2 kinase is phosphorylated. This leads to a balance between the induction of autophagy and the inhibition of peptide elongation.[34]

Although initially controversial, there is now good evidence that myocardial ischemia activates the tumor suppressor gene *p53*, and that *p53* contributes to myocyte cell death, since post-ischemic death is reduced in *p53* null mice.[35–37] There is also evidence that *p53* can regulate autophagy, although in a context-dependent manner. In cells with low basal autophagy exposed to nutrient starvation, *p53* enhances autophagy; but in cells with high inherent autophagy, *p53* does the opposite when nutrients are reduced.[38] These effects are mediated, at least in part, through LC3 and by *p53*-dependent activation of mTOR suppressors such as AMPK beta and PTEN, and require DRAM.[39]

In addition, mTOR can influence autophagy through *p53* and the *p53* family member, *p73*. Thus, mTOR positively regulates *p53*, and negatively regulates *p73*, by affecting their transcriptional actions on autophagy genes such as *Atg5* and *Atg* 7.[40,41]

Autophagy is also modulated by other signaling pathways that are independent of mTOR. Apoptosis during myocardial injury is known to be regulated by microRNAs, such as miR-1, miR-133, miR-199, miR-208, miR-320, miR-21, and miR-204, etc., that interact with the 3' ends of mRNAs, resulting in mRNA inactivation and/or degradation and thereby negatively regulating protein expression. For example, miR-204 has an anti-apoptotic effect during I/R injury by controlling LC3-II protein expression rather than LC3-I protein regulation through the 9 complementary bases.[42] Recently, a research team assessed the effects of PPAR-γ activation on myocardial miRNA levels and the role of miRNAs in I/R injury. It was found that miR-29 downregulation by antisense inhibitors and a PPAR-γ agonist protected against myocardial I/R injury.[43] The experiments were conducted on rat heart after a week-long pioglitazone (PIO) administration utilizing miRNA arrays. A marked reduction in the levels of miR-29a and c was found by Northern blotting. Blocking the effects of PIO and rosiglitazone on expression of miR-29 by the antisense PPAR-γ inhibitor GW9662 in H9c2 cells downregulated miR-29 and decreased caspase-3 activity, resulting in increased myocyte survival. Both infarct size and apoptosis rate of the IR myocardium diminished with inhibition of miR-29.[43]

Resveratrol (3,4′,5-trihydroxystilbene), and its commercial formulation longevinex, are potent cardioprotective agents. They suitably and significantly modulate the expression profile of miRNAs in rats pretreated for about three weeks, thereby leading to cardioprotection against I/R injury. Nearly 25 miRNAs, including those targeting the genes of sodium-potassium ion, metal ion binding, transcription factors and myocardial protection, are shown to be differentially expressed in treated hearts.[44] A unique signature of miRNA profile is observed in control hearts pretreated with resveratrol or longevinex.

Zhang and colleagues have demonstrated that prolonged hypoxia induces mitophagy to promote mouse embryonic fibroblast (MEF) survival.[45] This process was mediated via activation of hypoxia inducible factor-1 alpha (HIF-1 alpha) and required the presence of BNIP3 (Bcl-2 and nineteen-kilodalton interacting protein-3) and autophagy proteins including Beclin 1 and Atg5.[45] A recent study has shown that under starvation conditions, the p110-beta catalytic subunit of the Class I PI3-K could directly promote the stimulation of autophagy in MEFs, and in the liver and heart through its association with the Class III PI3-K initiation complex. This autophagy-promoting role of p110-beta was independent of its kinase activity and Akt activation.[46] According to its downstream target, the growth factor-mediated activation of the Ras signaling pathway either

activates or inhibits autophagy. In NIH3T3 mouse fibroblasts, activation of the Class I PI3-K cascade by Ras results in inhibition of autophagy.[47] In contrast, the Ras-mediated activation of the Raf-1–MEK1/2–ERK1/2 signaling cascade in response to amino acid depletion promotes autophagy.[48] Finally, death-Associated Protein kinase (DAPK) and DRP-1, two members of the Ca^{2+}/calmodulin-regulated Serine/Threonine death kinase family, were found to be necessary for the induction of autophagic programmed cell death in HeLa and MCF-7 cancer cells subjected to multiple stimuli including amino acid depletion.[49] In a recent study, DAPk was shown to promote the initiation of autophagy by phosphorylating Beclin 1, which is subsequently released from the anti-apoptotic Bcl-2/Bcl-X_L complex.[50,51]

Recent studies have also investigated whether the transcription factor NF-κB modulates myocyte survival post I/R through effects on autophagy. In a rabbit model in which I/R was induced by left circumflex coronary artery ligation followed by reperfusion, there was high expression of ROS and Beclin-1 together with the p65 subunit of NF-κB.[52] Furthermore, NF-κB inhibition resulted in reduced expression of Beclin-1 along with reduced autophagy in the area at risk; which conclusively led to a decrease in the extent of area at risk.

Parenteral administration of hydrogen sulfide in pig hearts subjected to I/R has also been reported to modulate infarct size and autophagy. Two different regimens of parenteral administration (infusion and bolus) of hydrogen sulfide were compared to a placebo.[53] Infusion group was found to have a significantly reduced myocardial infarct size ($P < 0.05$), with lower levels of mTOR and apoptosis-inducing factor ($P < 0.05$). Bolus group was found to show increased expression of phospho-p44/42 MAPK extracellular signal-regulated kinase, along with lower levels of Beclin-1 ($P < 0.05$). Overall, animals treated with hydrogen sulfide had reduced myocardial necrosis after I/R, with infusion administration observed to be better than bolus alone.

Autophagy plays a crucial role in the heart.[54] The occurrence of autophagy in cardiac myocytes was reported for the first time in 1976 by Sybers and co-workers.[55] Because cardiac myocytes are terminally differentiated and unable to renew themselves, they rely on autophagy to maintain their viability and functionality. Cardiac myocytes have a high requirement for ATP which is reflected in their abundance of mitochondria. The presence of mitochondria in autophagosomes in cardiomyocytes exposed to various stress conditions has been reported by several groups.[55–57] The significance of autophagy in the healthy heart has also been emphasized in several studies. A defect in the degradation phase of autophagy resulting from a deficiency of LAMP2, a lysosomal membrane protein, induces severe cardiac dysfunction, both in patients and in mice.[58,59] Most importantly, deficiency in Atg5 expression in the heart of adult mice resulted in the development of left ventricular dilation, cardiac hypertrophy and contractile dysfunction.[60] Autophagy has been shown to be altered in various cardiac disorders including ischemic heart disease, cardiomyopathies and myocardial hypertrophy.[61]

3 Autophagy in *in vitro* models of myocardial I/R injury

The role of autophagy in the pathogenesis of myocardial I/R injury has been investigated in various experimental models (*in vitro*, *ex vivo* and *in vivo*) and species (rat, pig, rabbit and mouse). The majority of these studies have demonstrated an increased presence of autophagic vacuoles in the heart. In addition, conclusions drawn from these studies portrayed autophagy either as a cardioprotective mechanism or as a contributor to cell death. The heterogeneity of the experimental models and their associated inherent variability make it difficult to attribute a rational justification of these conflicting results.

There is enough data available to support the up-regulation of autophagy following I/R in cardiomyocytes. However, differential induction of autophagy in terms of the extent of the ischemic insult has been reported in cultured H9c-2 cells by Loos, *et al*.[62] In this study, metabolic inhibition with sodium dithionate alone or together with 2-deoxy-D-glucose was used to inflict 'moderate' or 'severe' ischemia, which resulted in apoptotic and necrotic cell death without any evidence of autophagy. Apoptosis and autophagic up-regulation were both initiated by 'mild' ischemia, which was achieved by using 2-deoxy-D-glucose alone in the ischemic buffer.[62] Hence, different degrees of ischemic insult inflicted through varying periods of coronary occlusion may influence the presence or absence of autophagy up-regulation.

Variables such as the intensity of the ischemic insult and the methodology used to assess the occurrence and the function of autophagy may further complicate the picture. The next section of this review offers a summary of the knowledge derived from studies of autophagy carried out in isolated cardiac myocytes.

3.1 Modulation of autophagy in isolated cardiac myocytes exposed to I/R

Great efforts have been directed towards the development of experimental conditions that recapitulate the

changes (extra- and intra-cellular) elicited in the heart by ischemia. Thus, the model of simulated I/R (sI/R) has been used by several groups and has proved valuable in identifying key biochemical and cellular events underlying the pathogenesis of myocardial I/R injury. However, this basic model can include a number of variations, which may influence the results obtained; for example, the use and formulation of the "ischemia buffer", the level of oxygen (anoxia, hypoxia), the duration of I/R, and the cellular model (primary cultures of isolated neonatal or adult cardiac myocytes, immortalized cardiac cell lines such as HL-1). The autophagic response of cardiac myocytes following I/R injury has been examined in an *in vitro* model of anoxia-reoxygenation (AR). An increase in autophagy was reported in rat neonatal cardiomyocytes[63] and H9c2 cardiac cells[64,65] exposed to 30 min of anoxia and 60 min of reoxygenation. It is important to note that the lack of oxygen is only one component of ischemia, and anoxia or hypoxia is not sufficient to recapitulate the conditions of ischemia at the organ level. Therefore, the data derived from this model have to be interpreted cautiously.

Several groups have used the model of sI/R to study the modulation of autophagy in isolated cardiac cells. Valentim, *et al*.[66] observed an increase in autophagosomes (acidic vacuoles positively marked for monodansylcadaverine or lysotracker) in primary cultures of both neonatal and adult rat cardiomyocytes exposed to 4 h of simulated ischemia (2.0% O_2) followed by 16 h of reperfusion. Autophagy is a highly dynamic process that can be altered at various stages. An increase in autophagosomes can result from two distinct phenomena: an up-regulation of autophagy or a blockade of the degradation step of autophagy. To discriminate between these possibilities, it is essential to block the degradation phase of autophagy. Indeed, the rapid turnover of autophagosomes is a caveat when assessing autophagy. Regardless of the technique used to monitor autophagy, the abundance of autophagosomes measured at a given time does not reflect the flux of autophagy in the cell. Thus, to circumvent this issue it is essential to block the degradation of autophagic vacuoles, resulting in a time-dependent accumulation of autophagosomes in the cell. This can be achieved by neutralizing the lysosomal pH with Bafilomycin A1 (Baf A1), a proton pump inhibitor which results in the blockade of the autophagosome-lysosome fusion. In addition, inhibitors of lysosomal proteases (e.g., E64D and pepstatin A methyl ester) can be used to prevent the degradation of LC3-II when the latter is used as a marker of autophagy. The Gottlieb group demonstrated the importance of this approach. Indeed, they have shown that HL-1 cells overexpressing GFP-LC3 exposed to 2 h of simulated ischemia in the absence of oxygen exhibited a low level of autophagy. The subsequent reperfusion of ischemic cardiac cells induced a partial reactivation of the autophagic process shown by the increase in autophagosomes after 1.5 h[57] and 3 h.[67] The addition of Bafilomycin A1, E64D and pepstatin A methyl ester to the cardiac cultures at the onset of reperfusion allowed them to demonstrate that the increase in autophagosomes following sI/R resulted from an impairment in the clearance phase of autophagy, due to the presence of non-functional lysosomes. HL-1 cells exposed to 2 h of simulated ischemia (0.5% and 2.0% O_2) followed by up to 4 h of reperfusion exhibited low levels of autophagy as assessed by LC3-II Western blotting in presence of Baf A1. In an observation made by Hamacher-Brady and colleagues, autophagy was shown to be completely blocked in HL-1 cells exposed to 2 h of simulated ischemia in absence of oxygen.[67] This supports the notion that oxygen acts as a pivotal factor for autophagy modulation in ischemia.

As demonstrated by the aforementioned studies, an increase in autophagosomes can be easily misinterpreted, and the introduction of pharmacological agents such as Bafilomycin A1 or chloroquine in the experimental design is indispensable. The majority of the studies showing an increase in autophagy in the heart or isolated cardiomyocytes following I/R have not used this approach and thus failed to identify the cause of this effect (up-regulation or blockade of autophagy). In addition, as discussed, the severity of ischemia and its duration are key factors in the nature of the autophagic response during I/R.

3.2 The role of autophagy in I/R injury in isolated cardiac myocytes

Various approaches have been used to elucidate the role of autophagy during myocardial I/R injury *in vitro*. The blockade of autophagy is commonly achieved by inhibiting proteins that play a critical role in the initiation step of autophagy such as class III PI3K. Thus, to functionally implicate autophagy in the pathogenesis of myocardial I/R injury, several groups have used pharmacological agents such as 3-methyladenine (3-MA) and Wortmannin. However, considering the lack of specificity of the aforementioned inhibitors, the use of a gene-based approach to selectively modulate the expression or activity of initiators of autophagy has also been recommended. For example, down-regulation of Beclin 1 expression by RNA interference and over-expression of $Atg5^{K130R}$, a dominant negative mutant of *Atg5* were used by several investigators. However, it is worth mentioning that *Atg5* as well as Beclin 1 have been shown to play a role in apoptosis. Valentim and colleagues have shown that the inhibition of autophagy achieved by 3-MA

treatment or down-regulation of Beclin 1 reduced both the percentage of myocytes undergoing autophagy and cell death after sI/R.[66] They concluded that autophagy was detrimental to cardiac cells during I/R.[66] In contrast, Hamacher-Brady and colleagues have reported a cardioprotective effect of autophagy against sI/R on several occasions.[67] They have shown that treatments with either 3-MA or wortmannin, down-regulation of Beclin 1 or over-expression of $Atg5^{K130R}$ all sensitized HL-1 cardiac cells to apoptosis induced by 2 h of simulated ischemia followed by 1.5 h and 3 h of reperfusion. To gain some insights into the role played by autophagy during myocardial I/R injury, the investigators[8,66,67] used a second approach. The latter relied on the use of ischemic pre- and post-conditioning strategy or drugs mimicking the protective action of pre- and post-conditioning. The rationale behind this approach is that if a cardioprotective strategy/drug decreases cardiac cell death induced by I/R through inhibition of autophagy, it can be inferred that autophagy contributes to cardiac cell death, and vice versa. This approach allowed the investigators to not only delineate the nature of the role played by autophagy during myocardial I/R injury, but also to gain some insights into the molecular mechanisms underlying the cardioprotective action of these strategy/drugs against I/R injury in relation to autophagy. For example, the treatment of neonatal cardiac myocytes with urocortin prior exposure to sI/R resulted in a concomitant decrease in autophagy and cell death.[66] In contrast, Khan and colleagues have demonstrated the protective role of autophagy by using rapamycin, a drug that activates autophagy through inhibition of mTOR.[68] The treatment with rapamycin (25–100 nmol/L) of mouse ventricular cardiomyocytes reduced both necrosis and apoptosis induced by 40 min of simulated ischemia (1%–2% O_2) and 1 h and 18 h of reperfusion.[68] The preconditioning mimicking agent, 2-chloro-*N*(6)-cyclopentyl adenosine (CCPA, a selective agonist of the adenosine receptor A1) has also been shown to mediate its cardioprotective action through autophagy as it failed to protect HL-1 cells and adult rat cardiomyocytes against the injurious effect of sI/R when autophagy was blocked by overexpressing $Atg5^{K130R}$.[67]

4 Summary and perspective

The controversy surrounding autophagy (magnitude and role) in the context of myocardial I/R injury may stem from several factors. These include the variability in the experimental models, and as previously discussed, the methodology used to study autophagy–which also plays a critical role. From reviewing the literature, it is likely that autophagy can play both, a protective and a deleterious role in myocardial I/R injury and that which of these predominates may all depend on the experimental context.

The role of autophagy in myocardial I/R injury appears to be dependent on the severity of ischemia and its duration. In addition, the level of activation of autophagy may dictate the nature of its role. Under stressful conditions, autophagy is activated as an adaptive response to maintain the survival of cells. However, if the cellular stress is not manageable and elicits damage that is beyond repair, the (over) activation of autophagy may drive cells to die. In the context of myocardial I/R injury, the extent of the damage caused to the heart is highly dependent on the severity of the ischemic insult.

Although the reasonably sound understanding of the molecular pathways of autophagy make it theoretically attractive as a therapeutic target, the relevance of knowledge obtained from the experimental models to the ischemic human heart remains unclear. Presently, our knowledge is insufficient to support the notion that autophagy may represent a potential therapeutic target. Thus, to precisely define the role of autophagy in myocardial I/R injury, it is necessary to establish and standardize the experimental models (*in vivo, ex vivo* and *in vitro*) that recapitulate various degrees of ischemic stress.

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