

Review Article

Autophagy, a process within reperfusion injury: an update

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Abstract: Autophagy is an important constitutive intracellular catalytic process that occurs in basal conditions, as well as during stress in all tissues. It is induced during cellular growth, tissue differentiation and metabolic demands. The regulated expression is cytoprotective while its deregulation leads to varieties of diseases. It plays a vital role in ischemic heart disease, being beneficial and adaptive during ischemia while detrimental and lethal during reperfusion. Reperfusion injury is the consequence of this deregulated autophagy and the motive of its persistence during reperfusion is still obscure. A long standing debate persists as to the dual nature of autophagy and defining its clearer role in cell death as compared to the widely studied process, apoptosis. Despite the progresses in understanding of the process and identification of critical mediators, there is no therapeutic strategy to address its final outcome, the reperfusion injury. This lack of effective therapeutic strategies has even questioned the validity of the process as a single entity. We still continue to witness the devastation with standard cure of reperfusion. In this article, we review the process, highlight reperfusion injury and outline important studies being conducted for the prevention of reperfusion injury and offer cardio-protection.

Keywords: Autophagy, cardiomyocytes, myocardial ischemia, myocardial reperfusion, Ischemia/reperfusion injury

Introduction

Ischemic heart disease (IHD) is the leading cause of death worldwide [1] and continues to rise. The global burden of disease studies, carried out by the World Health Organization and the World Bank projects the number of deaths to reach 11.1 million (from 6.2 million in 1990) by 2020, which can be seen on their website [2]. The subsets of IHD; angina pectoris, unstable angina and myocardial infarction (MI) differ in the severity, frequency and duration of sign and symptoms. These diseases share a common pathology of atherosclerotic plaque build-up in the intima of coronary artery and clinically manifest according to the severity of coronary obstruction. MI, commonly called as 'heart attack' is the most severe form of IHD. The standard mode of cure is re-establishment of coronary perfusion (reperfusion) by thrombolytic therapy, primary percutaneous intervention (PPCI) or coronary artery bypass grafting (CABG). This has been a revolutionary strategy that has limited death and morbidity associat-

ed with MI, but reperfusion gives rise to a newer form of ailment called as reperfusion injury (RI) that is responsible for post reperfusion cardiac morbidities and heart failure. Moreover, a rough estimate states that there are about 1.6 million patients that undergo some form of reperfusion therapy in hospitals and specialist clinics in the western world alone [3] and the total number worldwide would be alarming. These data cannot be taken as a reference but the severity can be true. Validation of newer therapeutic targets and strategies, which could limit RI associated morbidities, is still an unmet clinical need.

Autophagy is functional from the very embryonic period [4] and plays a fundamental role in growth and differentiation of body tissues. Its dynamic role during muscle development in vital tissues like the heart, diaphragm and alveolar cells makes it a survival ensuring process [5, 6]. It is responsible for nutrient homeostasis, energy salvage and degradation of old, malfunctioning organelles within a cell. Additionally, it has a cytoprotective component which fights

Table 1. Role of autophagy in health and development

Biological effects	Cytoprotective
<i>Embryo</i>	Pre-implantation of fertilized oocyte, elimination of maternal mRNAs [15], elimination of paternal mitochondria [16], gastrulation, stem cell differentiation, placental development, organogenesis, differentiation of erythrocytes, adipocytes, lymphocytes [17], neuron [18]
<i>Birth</i>	Nutritional deficiency on initiating sucking. Fetus to neonate transition [5]
<i>Adult</i>	Adaptations to exogenous stimuli (Hormonal, growth factors, cell density loads, nutrient depletion, oxidative stress)
<i>House keeping/quality control</i>	Disposal of misfolded and damaged proteins aggregates [19] and dysfunctional organelles [20]
<i>Nutrient Recycling</i>	Organelle degradation end product–glucose, amino acids, fatty acids and nucleic acids recycling.
<i>Fighting pathogens</i>	Identification of ligands of different bacteria and viruses and engulfing them for degradation.
<i>Immune protective</i>	Thymic selection, Effector of TLR signaling, Effector of Th1/Th2 polarization, Antigen presentation
<i>Longevity</i>	Anti ageing
<i>Defective autophagy</i>	Neurodegenerative diseases-Huntington's disease, Parkinson's disease, Alzheimer's Disease, Lafora Disease, Lysosomal Diseases Cancer Liver disease-Hepatocellular carcinoma, Hepatitis, Fibrosis Metabolic syndromes-Diabetes, Obesity, pancreatitis Infectious disease Cardiac and musculoskeletal: Cardiomyopathy, hypertensive heart failure, Hypertrophy, ischemia/reperfusion injury, Pompe's Disease Inflammation-Crohn's Disease

the invading pathogens and provides immune protection [7]. In contrast to these salutary survival promoting aspects, autophagy is also a type II programmed cell death and can initiate cell death in different circumstances. This delirious autophagic response have been attributed to many diseases and disorders such as –neurodegenerative diseases [8], cancer [9], liver diseases [10], cardiac diseases [11], Metabolic syndromes [12], ageing [13], inflammations [14]. From the very inception, the process of autophagy has faced controversies regarding the cell death process and has been subjected into enormous cross talks with apoptosis, another well studied cell death process type I. Advances in research and medicine have established the process of autophagy, with its dual nature as a separate functioning entity with a purpose. The purpose for survival is well understood but the purpose for death is unknown. Numerous studies have been conducted and are being conducted with drugs targeting different pathways, which will be outline later in the text. Listed below in **Table 1** are the overall effects of autophagy in basal conditions, condi-

tions of stress and when the process gets deranged.

The direct contribution of the process of autophagy in the development of reperfusion injury has been established and numerous researches aimed on minimizing this injury have been conducted and are being conducted.

This article provides an update on the process of autophagy and reperfusion injury as well as outlines significant studies and clinical trials at present.

Literature search methodology

For this review, the PubMed database was searched for articles concerning autophagy and its role in the development of ischemia, reperfusion injury which were published in English before July 2014. We used the search terms “Autophagy” and “Ischemia-Reperfusion Injury”. Clinical studies were considered if they evaluated the association of autophagy with the pathogenesis, pathological features, phar-

macological targets in ischemia and reperfusion (I/R) injury, accordance to Author's judgment.

Characteristics of autophagy

Overview of autophagy

Discovery of the process, word: The phenomenon of 'self-eating' was first observed by Ashford and Porter during 1962 in the rat liver cells [21]. The word 'autophagy' was coined by Belgian biochemist Christian de Duve in 1963 [22], which is Greek in origin and means "to eat" (phagy) "oneself" (auto). The cell gets rid of cytoplasmic contents, long lived proteins or even organelles by means of lysosomes, also known as-type II programmed cell death. Depending upon the route of cargo transport, autophagy is of three types; macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) [23]. Macroautophagy, hereafter termed as 'autophagy' engulfs the cytoplasmic content into autophagosomes while microautophagy utilizes lysosomal membrane which engulfs the cytoplasmic content. During CMA, a chaperone identifies a specific protein peptides or polypeptides in the cytosol. The autophagosomes and the chaperone transports the content for degradation to the lysosomes while microautophagy requires no transportation. Autophagy has been observed in invertebrates as well as in mammals. Many of the genes and proteins of autophagy were first discovered in yeast and have been named as autophagy related genes (Atg) and their respective proteins [24]. By now, more than 30 autophagy related genes (Atg) have been identified and many of them have homologues in mammalian cell [25].

Autophagy components: The process of autophagy comprises of triggers and stimulators, sensors and genetic regulators, autophagy related genes and proteins and the final effectors.

Signaling pathways

Trigger: Almost any signal can be a trigger for autophagy, some activating the pathway and some suppressing the pathway. By far, energy depletion and oxygen deficient environment are the most powerful triggers for stimulating autophagy, while the reverse environment

inhibits autophagy. Similarly, the growth factors, hormones, receptors with cytokine activities, receptors with tyrosine kinase activities and receptors that recognize pathogen ligands can also activate autophagy. The low energy sensors activate the adenosine-monophosphate-activated protein kinase (AMPK) pathway or the Beclin-1/class III phosphatidylinositol kinase (PI3K) pathway while hypoxia induces transcriptional factors and mediators for autophagy. The high energy sensor is mammalian target of rapamycin (mTOR) which on the other hand is a negative regulator of autophagy. In addition to the nutrient status within the cell, activation of cell surface receptors with tyrosine kinase activities such as endothelial derived growth factor receptor (EGFR), neurotrophins (TrkA/B), fibroblast growth factor receptor (FGFR), platelet derived growth factor receptor (PDGFR) through adaptor protein GRB2, leads to series of phosphorylations and activations of Ras proteins and later Raf protein kinases. This Raf further activates mitogen activated-protein kinases (MAPK) like- extracellular signal regulated kinase 1/2 (ERK 1/2), phosphoprotein 38 (p38), c-jun N-terminal kinase (JNK), which is a positive regulator of autophagy process [26, 27]. Similarly, invading pathogen express ligands that are recognized by toll like receptor (TRAIL-R), esp. TLR 7 which activate autophagy [28]. In the same manner, tumor necrosis factor receptor (TNF-R), that is activated by various cytokines can either induce survival autophagy via tumor necrosis factor receptor type-1 associated death domain protein (TRADD), ribosome-inactivating protein (RIP), tumor necrosis factor receptor-associated factor 2 (TRAF2), inhibitors of apoptosis (cIAP1) to nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) or can induce death autophagy via fas-associated protein with death domain (FADD) or procaspases 8 and 10 [29]. Additionally, BCL-2/adenovirus E1B 19kDa interacting protein (BNIP), death associated protein kinase (DAPK), dynamin related protein 1 (DRP-1) [30-32] have also been identified to stimulate autophagy in tissues. Here, we discuss autophagy in a cardiomyocyte in different settings.

Inhibitory pathway: Phosphatidylinositol 93.4.5)-triphosphate kinase class I (PI3K-I)-Akt-mTOR axis: With nutrient abundance that is modulated by signals from PI3Ks of cytokine

receptors, G protein-coupled (GPC) receptors, growth factor receptors and integrin receptors, the PI3K-Akt-mTOR axis gets activated. The Akt exerts an inhibitory action on the assembly of tuberous sclerosis complex 1 and 2 (TSC1/TSC2), that relieves the inhibition of Rheb on mammalian target of rapamycin kinase 1 (mTORC1) thereby activating it. The mTORC1 [33] negatively regulates autophagy by inactivating Unc-51 like autophagy activating kinase 1 (ULK1 kinase) that consists of ULK1, Atg 13 and Atg 17.

one hand initiates autophagy by stimulating p27 gene that starts manufacturing autophagic machineries [35]; On the other hand it inhibits mTORC1 by dissociating TSC1 from TSC2, rendering it inactive. Similarly, hypoxia targets the hypoxia induced hypoxia inducible factor Hif-1-protein kinase C delta PKC δ -JNK1 axis [36]. This axis promotes interaction of Beclin-1 and phosphatidylinositol 3 kinase class III (PI3K III), an essential step in the phagophore induction. Additionally, this interactions is also induced or promoted by nutrient depletion, especially amino acid depletion [37] (notably leucine depletion in cardiac tissue [38]) and molecular mediators like Ca²⁺ and reactive oxygen species (ROS). Moreover, organelle stress itself can directly activate or generate mediators that influence both pathways. For instance, DNA damage and oxidative stress can inactivate mTORC1 through sestrin, a protein induced by p53 gene, which culminates in mTORC1 inhibition [39]. Similarly, endoplasmic reticulum (ER) stress can activate endoplasmic reticulum

kinase (PERK), which through a series of activation of eukaryotic translation initiation factor 2 α (eIF2 α) kinase Fcn2 (another energy sensor), induces its downstream target of Gcn4 [40], and can activate autophagy related genes to initiate the manufacturing of autophagic proteins [41]. Likewise, another protein, regulated in development and DNA damage responses-1 (REDD-1) can be activated by hypoxia and can directly disrupt the assembly of TSC1 and TSC2 [42]. Moreover, oxidative, genotoxic stress and organelle stress can induce nuclear transcription factor p53 and activate autophagy by transactivating AMPK [43], while the cytoplasmic p53 protein can inhibit autophagy by activating mTORC1. The key steps and interactions of pathways of autophagy is outlined in **Figure 1**.

Reperfusion: It is logical at this point to state that besides the quality and quantity of previous blood flow, perfusion and reperfusion share the same physiology and should follow the same inhibitory pathway of autophagy. Surprisingly, with unexplained processes and undiscovered molecular mediators, Beclin-1 and microtubule associated protein 1 (MAP 1) light chain 3 (LC3), which is a marker of autophagosome maturation is reported to be elevated, signifying the ongoing autophagy and cellular damage [44]. This form of cellular death goes against the theoretical principles of survival due to nutrient and oxygen abundance and has indeed challenged reperfusion strategy in different fields, notably cardiology.

Key mediator molecules

Reactive oxygen species (ROS) is well known that hypoxia, organelle stress (especially the mitochondrial and endoplasmic reticulum [45]) and dysfunctional enzymes during myocardial ischemia generate tremendous amount of ROS. ROS is a powerful activator of starvation-induced autophagy [46], that causes oxidative damage to the organelles and lipid peroxidation of lipid membranes especially in the mitochondria, which leads to fatal cell death [47]. Generation of ROS continues or increases from the mitochondria even with the commencement of myocardial reperfusion by a mechanism called ROS-induced ROS release when electron transport resumes [48]. Besides cellular damages, ROS is also responsible for

post-ischemic contractile dysfunction, called 'myocardial stunning' where the heart muscles show reduced contractions after reperfusion [49] although it is reversible.

Calcium is another molecule that gets accumulated and propagates ischemia/reperfusion (I/R) injury in the cell. The lack of ATP with the resultant anaerobic glycolysis generates lactic acid that makes the environment acidic. To neutralize this environment initially sodium comes inside the cell in exchange of hydrogen ion and with time to get rid of excess sodium from the cell, calcium comes in and sodium ion is extruded [50]. This becomes the last step that the cell can offer to maintain homeostasis. With no reversal of ischemia, piling up of calcium ensues. Simultaneously, the ER stress also spills Ca²⁺ into the cytosol. This calcium then activates ROS and induces opening of the mitochondrial permeability transition pore (mPTP), cessation of electron transport chain, loss of membrane potential, matrix swelling, inner membrane remodeling and finally outer membrane rupture with subsequent release of mitochondrial cytochrome C and other apoptogenic proteins [51]. This leads to intracellular catastrophe. Reperfusion, adds more and further brings in calcium that further induces ROS and exacerbate damage by disrupting the plasma membranes, damage to the sarcoplasmic reticulum and mitochondrial re-energization [52].

Beclin-1 protein plays a crucial role in the induction and maturation of autophagosome. It is a mammalian ortholog of yeast Atg 6. Beclin-1 is normally in an inhibited state being bound to Bcl-2 protein (anti-apoptotic), and localized in the endoplasmic reticulum. This molecule is seen to demonstrate a dual nature of action in experimental models. It is essential for cellular survival in mice L292 fibroblasts [53] and murine embryonic fibroblasts (MEFs) [54]. On the contrary, it is a necessary component of autophagy mediated cell death and this has been documented by genetic suppression of Beclin-1, that resulted in augmentation (rather than impairment) of cellular survival in cardiomyocytes [55]. The exact trigger for Beclin-1 activation is unclear but some studies have identified intracellular amino acids depletion [19], and activated ROS [56] as a strong stimulus. Similarly, nutrient depletion itself weakens the bond between Beclin-1 and Bcl-2 thus liber-

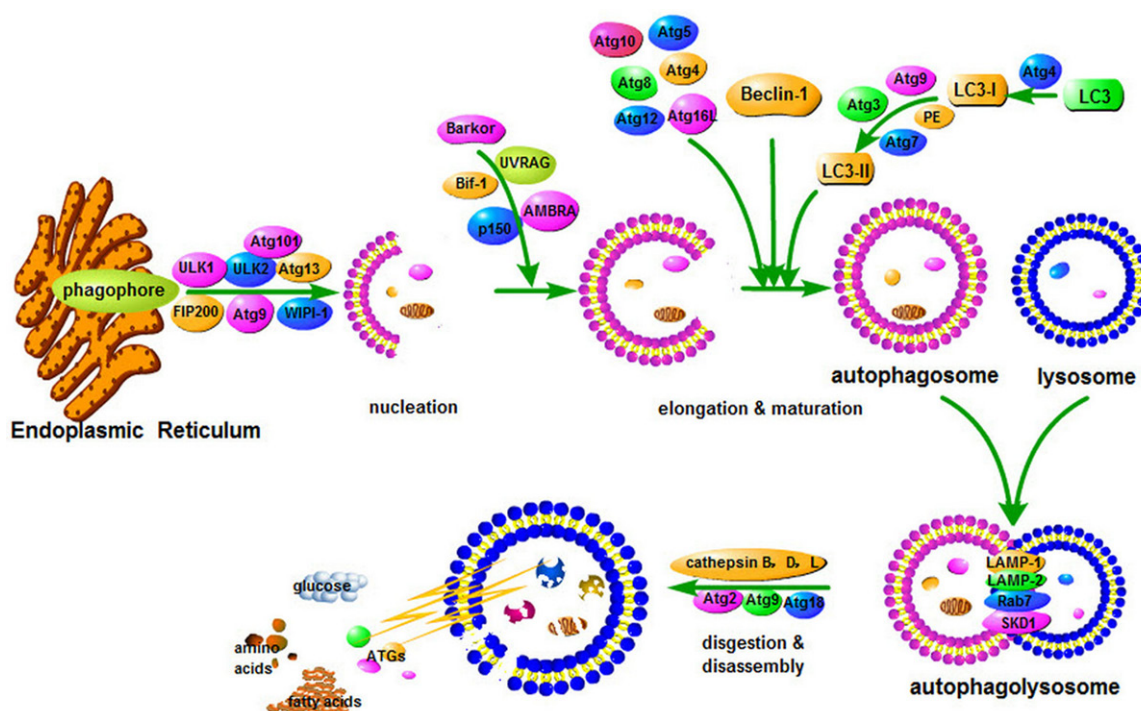


Figure 2. The autophagic (macroautophagy) pathway for cellular degradation. The triggers initiate phagophore which starts sequestering cytoplasmic content. Phagophore then nucleates, elongates and matures. A mature autophagosome fuses with the lysosomes where degradation of the sequestered cytoplasmic content occurs. The end product of degradation - glucose, amino acids, fatty acids, nucleic acids along with the Atg proteins are released into the cytosol for re-use.

ating Beclin-1(which is its active form). The reason of expression of Beclin-1 during reperfusion is still a mystery but some undiscovered mediators may be activating Beclin-1 directly, may be weakening the Beclin-1-Bcl-2 bond or may be inhibiting the Bcl-2 molecule itself. These hypotheses need more research and validity. Moreover, Beclin-1 inhibition or activation by Bcl-2 protein has been seen an indirect control of autophagy by apoptosis. Thus, Beclin-1 proteins role at the juncture of cellular death or survival and autophagy or apoptosis has invited numerous cross talks among scientists. Identification of the 'switch' that turns one process on and the other off and the vice versa has been a platform of researches.

The release of Bcl-2 homology domain 3 (BH3)-only proteins occurs in response to hypoxia and release of ROS. It is the downstream target of HIF-1, and can trigger autophagy by controlling the activities of pores and channels in the outer mitochondrial membrane, inducing mPTP opening and mitochondrial damage [57]. Not only during autophagy, but its role on initiating apoptosis mediated cell death in ventricular myocytes [58] has also been documented.

Some of the studies have also reported that BNIP protein not only influences but also up regulates autophagy, the purpose being for the removal of damaged mitochondria to confer cardioprotection [59]. These contrasting roles of BNIP protein during autophagy has also been a subject of researches and crosstalks. Till date, 6 BH3 proteins (BNIP3, PUMA, Bid, Bad, HGTD-P, Noxa) have been identified that take part in ischemia-reperfusion death pathways in heart and/or brain [46].

Autophagosome formation and execution

As mentioned earlier, any form of stress triggers autophagosome formation, which fuses with the lysosome with ultimate degradation of the sequestered material through the cytoplasm to vacuole (Cvt) pathway for degradation in autophagy [60]. Here, we outline only the molecules involved in the formation and execution of autophagy machinery, as the entire steps, interactions and the discussion is beyond the scope of this article.

Pre induction and induction: The initiation of autophagosome formation starts with protrusion

sion of a phagophore where a portion of cell membrane cups off from the endoplasmic reticulum [61, 62]. It is mediated by different inducers with their own pathways. The identified inducers of autophagy in mammals are-ULK1, Unc-51 like autophagy activating kinase 2 (ULK2), 200KD focal adenosine kinase (FAK) family kinase-interacting protein (FIP200), Atg 13, Atg 101, Atg 9 and WD repeat domain, phosphoinositide interacting 1 protein (WIPI-1) [63, 64].

Vesicle nucleation: Manufacturing of a double layered membrane starts at the phagophore which is a complex process and occurs with the interaction of Class III PI3K and beclin-1 along with contributions from beclin-1 regulators such as- Baf-1, BAX- interacting factor-1 (Bif-1), activating molecule for BECN 1 regulated autophagy protein 1 (AMBRA 1), ultraviolet-radiation resistance-associated gene (UVRAG) and p150 (mammalian homolog of yeast Vps15) [37, 65]. This structure starts engulfing the cytoplasmic content.

Vesicle elongation and maturation: Further elongation of the newly formed membranous vacuolar structure occurs which is aided by the Atg 12 conjugation system, Atg 8 conjugation system, Atg 5, Atg 10, Atg 16L, Atg 4, LC3, Beclin-1, Atg 7, Atg 3, phosphatidylethanolamine (PE) and Atg 9 [66]. A mature autophagosome is then formed.

Vesicle fusion with lysosomes: The newly formed autophagosome fuses with lysosomes by the interactions with lysosomal membrane proteins, lysosomal-associated membrane protein 1 (LAMP-1), lysosomal-associated membrane protein 2 (LAMP-2), the small GTPase Ras related protein 7 (Rab7) and an ATPase called SKD1 [67].

Digestion and Disassembly of Atgs: Finally, the degradation of the inner vesicle along with its contents occur which is dependent on a series of lysosomal/vacuolar acid hydrolases, including Cathepsins B, D and L [68]. The small molecular end-products of degradation, including amino acids, fatty acids, glucose and nucleotides, are released into the cytosol by permeases for reuse. Similarly, disassembly of the Atg proteins occurs that are mediated by Atg 2, Atg 9 and Atg 18 (**Figure 2**) [69].

I/R injury

It is clear that autophagic cellular damage during ischemia and reperfusion is the basis of reperfusion injury. This injury was firstly observed by Jennings et al. during 1960 in a canine model, who also noticed that reperfusion accelerated the process of necrosis formation [70]. Since then, numerous studies have been conducted that have broadened our understanding of the micro-process, the mechanical influences and the pharmacological interactions. This has exposed several possibilities in preventing RI. Many animal studies, in vitro studies and trials have been conducted so as to limit RI. So far the human trials and the enthusiastic search for therapeutic targets have been disappointing.

Reperfusion injury in a nutshell

With the commencement of reperfusion in cardiac tissues, there is increased blood flow that brings in nutrition, oxygen, polymorphonuclear cells (PMNs) and platelets to a previously ischemic area. The electron transport in the functional mitochondria resumes. ROS production starts immediately within the first few minutes [71] by the activated neutrophils [72] as well as by a process called ROS-induced ROS release, besides the continuous ROS release from previously damaged organelles. Researchers have found that ROS targets and activates Beclin-1, regulatory factors, activating factors and the endothelium by which the neutrophils and platelets are attracted [73], leading to *microvascular plugging*. In addition to the ill effects on the cellular and organelle morphology, ROS also damages the sarcolemma with liberation of calcium to the pool that is already filled up with calcium due to acidotic environment. The balance of calcium is altered and this disturbance in calcium homeostasis also plays an important role in the development of RI [74]. This calcium opens the mPTP [75] and releases caspases, second mitochondrial activator of caspase (SMAC), inhibitor of apoptosis (IAP) and endonucleases [76]. The calcium dependent protease (calpain I) are also activated that result in proteolysis of troponin I [77] with histological evidences of cell death [78]. The surviving myocardium too, is on the death pathway as demonstrated by the accumulation of autophagosomes and prominent up-regulation of lyso-

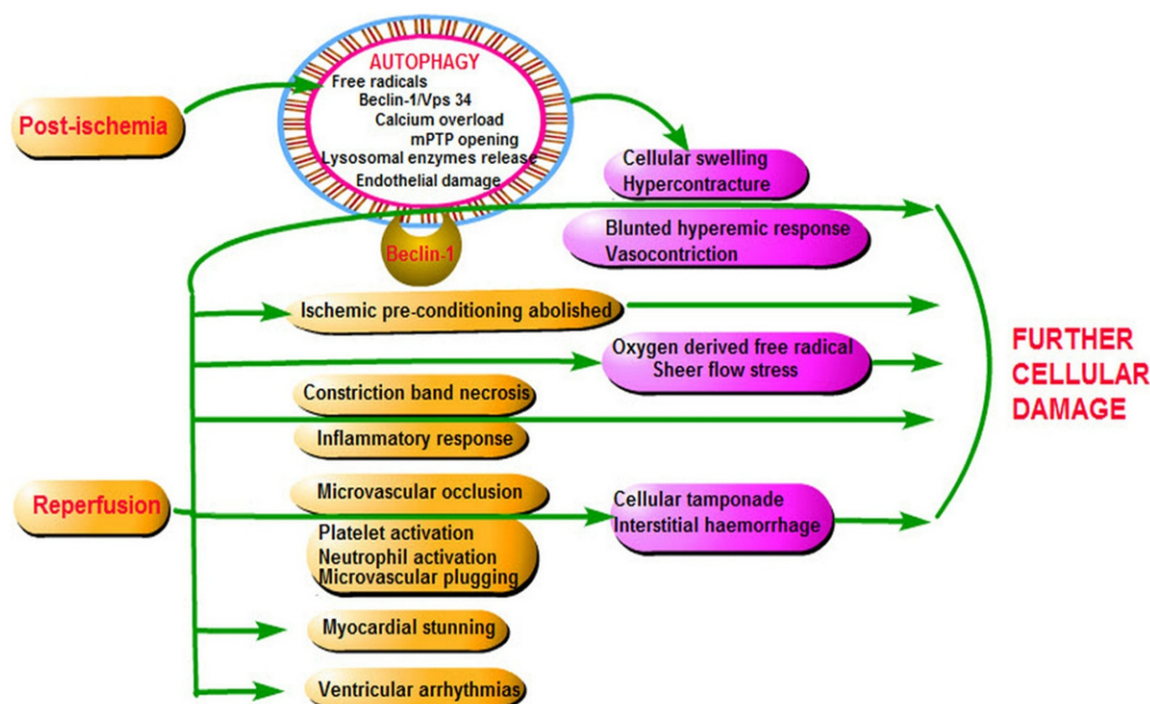


Figure 3. The overall effects of reperfusion on an ischemic cell. Reperfusion causes further cellular damage.

somal enzymes [79]. Besides these, a commonly observed feature of reperfusion is the rapid onset of contraction band necrosis [80] which marks the loss of plasticity and cell architecture. These processes are all interrelated and lead to reversible, treatable conditions such as ventricular arrhythmias [81] and myocardial stunning [82], but also can lead to irreversible condition like microvascular obstruction (MVO). MVO is defined as the “inability to reperfuse a previously ischemic region [83] grossly due to ultra structural damage to the microvasculature [84], endothelial abnormalities, neutrophil occlusion and erythrocyte stasis at the capillary level [75]. The presence of MVO has been demonstrated as sluggish coronary blood flow and impaired myocardial blush grade [85] even after successful reperfusion (PPCI). As a sequel of MI, the myocyte become edematous and necrotic, which can compress the microvasculature and increase distal vascular resistance, arteriolar spasm and progressive endothelial dysfunction [86]. This endothelial dysfunction in a severe case leads to endothelial damage and extravasation of blood into the interstitium. This form of intramyocardial hemorrhaging within the area of infarction has been demonstrated by cardiac MRI [87] after reperfusion. Additionally, if there were prior

exposures of sublethal ischemia, the myocytes and tissue in course time develop adaptations, a form of cardioprotection called the ischemic preconditioning [88], which with reperfusion gets abolished and the drive for survival in a compromised environment is lost. Besides these, the myocardial tissues are also unable to mount a reactive hyperemic response leading to increased vascular resistance in a previously ischemic and damaged tissue, due to loss of endothelial nitric oxide synthase (eNOS) that cannot generate Nitric oxide (NO), a physiological vasculature modulator. Another commonly noticed feature of MI is hyperglycemia (even in the absence of diabetes mellitus). It has been implicated with further deterioration of left ventricular functioning after reperfusion [89] and increased mortality after MI [90]. All these microscopic changes collectively give rise to ‘reperfusion injury’ or in severe cases the ‘lethal reperfusion injury.’ The process of superimposed cell death by reperfusion injury is outlined in **Figure 3**.

Autophagy and I/R injury in myocardium

Autophagy starts in the ischemic period and continues or even increases during the later stages of reperfusion [82]. It is an attempt to

survive the severely limiting conditions during ischemia, while it is a quest for survival following the overwhelming activation of cellular processes during reperfusion. The importance of I/R injury lies in the fact that each functional myocyte is vital for the normal cardiac functioning, as the turnover/regeneration is very slow; only about 1% of cardiomyocytes are renewed annually at the age of 25 which declines to 0.45%, at the age of 75 [91]. With this low and insufficient pool of myocytes at hand, reperfusion adds more insult as indirect evidences have demonstrated RI to contribute 40-50% of the final MI size [52]. The process of RI is not static, it is continuous as presence of MVO for up to 48 hours [92] and autophagy for up to 3 days in a previous ischemic area [93], have been documented. Opening an artery does not ensure perfusion as a phenomenon of vascular stasis called the 'no reflow' can occur which may last from 2 minutes to 3.5 hours [94]. The perfused myocardium can remain unresponsive (myocardial stunning) or even show irregular rhythm, contributed by persistent ROS activation, though reversible. RI can be perceived here, as a window that exposes the world of intracellular devastations of autophagy. The final outcomes of RI on the heart can be larger infarct size, lower left ventricular ejection fraction, adverse left ventricular remodeling and worse clinical outcomes [52].

Cross talk and the complex inter-relationship: autophagy and apoptosis

Apoptosis and autophagy as well as necrosis are the major processes of mammalian cell death. The former two differ from the latter by the feature of tissue inflammation. Apoptosis and autophagy both do not exhibit tissue inflammation and share many same death signals like ceramide, TNF-related apoptosis inducing ligand, FADD, DAPK, DAPK-1 [30-32, 95]. The interactions between the two processes are complex demonstrating agonism, antagonism or synergism. There are numerous literatures highlighting individual interactions; here we outline some important ones.

Autophagic regulation of apoptosis

The proteins involved during autophagy such as Atg5 and Atg 12 [96], Beclin-1 take part in the regulation of apoptosis. Studies have also demonstrated a mutual co-regulation between the

two where depletion of Atg 5 and Atg 3 was associated with suppression of caspase-8 activation and apoptosis [97]. Moreover, studies have also demonstrated that autophagy can regulate, sequester and eliminate caspase 8 [98], an effector of apoptosis.

Apoptotic regulation of autophagy

The apoptotic proteins such as Bcl-2, BCL-xL, and most of pro-apoptotic BH3 only proteins can initiate the process of autophagy. Similarly the negative regulation have also been observed in studies where the pro-apoptotic protein Bax [99] and Ste20-like kinase 1 (Mst1) [100] suppressed autophagy while the effectors of apoptosis, the caspases were also seen to inhibit autophagic proteins- Beclin-1, vps34, Atg3, Atg4D, AMBRA1 and p62 [101]. Studies have demonstrated cell death by apoptosis when autophagy is suppressed [79]. To add more, the inhibitor of apoptosis- the flc inhibitory protein (FLIP), has been found to be a negative regulator of autophagy [102].

With this diversity of interactions, some scholars have felt that the contributions of apoptosis on cell death far outweighs to that of autophagy, and have even suggested autophagy as being a smaller effector of the death process. Nonetheless, it stands out as a different entity with the hallmark of early organelle damage and late cytoskeleton derangement as compared to apoptosis where the reverse happens. There are distinctions in the histology and the roles played. Autophagy plays an added role of cellular renovation and sustenance [103] by means of 'self eating' as compared to the apoptotic role of 'self killing'. Further researches are warranted to answer the controversies related to autophagy mediated cell death, death signals generation, death signal interaction and its pharmacological and genetic regulation.

Experimental studies within the last 5 years

There are numerous studies that have been conducted all over the world in light to autophagy and reperfusion injury. Researches, literatures and reviews have highlighted the findings of effective pharmaceutical agents and the list seems encouraging. However, most of the experimental studies face a setback while relating into larger trials or human studies due to discrepancies in reliability and reproducibility.

Autophagy in ischemia/reperfusion injury

Table 2. Cardio protection delivering drugs, their interacting pathways on I/R injury: experimental/ animal study during the last 5 years

Target pathway	Pharmaceutical agents
PI3K/Akt	Salidroside (LY294002) decrease Bax and Caspase-3(2013) [104], Luteolin (poly-phenol flavonoid) inhibit Bax and apoptosis (2011) [105], PPAR- α activation as a preconditioning like intervention (2012) [106]/PTCA induced ischemic postconditioning (2012) [107], Ligustrazine (TMP) by itself and by phosphorylating eNOS to produce NO(2012) [108], Sitagliptin via GLP-1/GLP-1 receptor (2013) [109] Globular adiponectin modulate SERCA and suppress ER stress (2013) cardiomyocyte [110], Anandamide induction of HSP72 mediated by CB (2) and CB (1) receptors (2013) [111], Remote ischemic postconditioning by upregulating ALDH2 expressions (2014) [112]
PI3K/ Akt and ERK1/2 (RISK pathway)	Epigallocatechin-3 gallate [113]
PI3K/AKT and ERK1/2- MEK1/2	A3 adenosine receptor agonist, 2-CL-IB-MECA, inhibit caspase-3 (2014) [114]
PI3K/Akt and ERK1/2	Berberine (from Barberry) maintains intracellular calcium and prevents calpain activation (2012) cardiomyocytes [115]
PI3K/Akt and PKC pathway	Urantide (2012) [116]
PI3K and p38MAPK	Dobutamin-mediated heme oxygenase-1 induction (2013) [117], BNP post conditioning inhibit HMGB1, TNF- α and IL-6. (2014) [118]
PI3K/Akt/ERK	Intralipid (GSK-3 β) inhibit of mPTP (2011) [119] Achyranthes bidentata (herb) (2013) [120]
PI3K/Akt and ERK1/ERK2	Urocortin 2 autocrine/paracrine and pharmacological effects (2013) mouse ex vivo [121], GHRH through activation of RISK and SAFE pathway (2013) [122]
PI3K, Akt/PKC ϵ /MitoKATP/ROS signaling	Catestatin (2013) [123]
PI3K/Akt/mTOR/SV	Survivin potentiates the anti apoptotic effects of insulin. (2011) [124]
Arachidonic acid	t-AUBC inhibition of sHE enhances the activity of EETs (2010) [125]
Akt/PKC α / β	Aldolase reductase modulates GSK-3 β Phosphorylation (2012) [126]
PI3K/PKG/GSK3 β	ANP prevents mPTP opening by inactivating GSK3 β (2012) [127], Sufentanil postconditioning (2012) [128]
Activation of RISK/GSK-3 β and inhibition of p38 MAPK	Curcumin (turmeric) (2012) [129]
PI3K/Akt/GSK3 β	Dansen (salvia miltiorrhiza) (2013) [130]
PI3K/Akt and JAK2/STAT3 pathways	Fasudil inhibits ROCK, attenuates ER stress and modulates SERCA activity (2012) [131]
PI3K and NO	Therapeutic hypothermia at 34 °C (2012) [132]
PI3K/Akt/NDRG2	Potentiates the anti-apoptotic effect of insulin (2013) [133]
GSK3 β activation	Sevoflurane (2013) [134]
ROS signalling	Mitochondrial BK(Ca) (2013) [135]
Akt/PTEN pathway	Ischemic postconditioning-mediated miRNA-21 (2013) [136]
PI3K/Akt/FOXO3A/Bim pathway	Sodium tanshinone IIA sulfonate (2013) [137]
Paracrine pathway TIMP-1	Cardiac fibroblasts (2014) [138]
AMPK/Akt/JNK signaling activation	Labdane diterpenes (2011) [139] Rosiglitazone (2011) [140]
AMPK pathway	CTRP9 protein (2012) [141], Berberine (2012) [142], Macrophage migration inhibition factor, MIF (2013) [143], Omentin (2014) [144]
AMPK/Akt	D-dopachrome tautomerase enzyme (2014) mice [145], Antithrombin (2014) [146]
AMPK	Propofol inhibition, phosphorylation of mTOR (2010) [147]
Beclin-1	Ginsenoside Rg1 (2012) [148]
Inhibition of activation of AMPK α , Beclin-1, activation of mTOR, decreased level of LC3B-2	Trimetazidime (2010) [149]
K ⁺ channel opener	Nikorandil (2011) [150]

Source: PubMed. Abbreviation: Akt/PKB: Ak strain transforming/protein kinase B, ALDH2: aldehyde dehydrogenase 2, ANP: atrial natriuretic peptide, BNP: brain natriuretic peptide, CB: cannabinoid receptor, CTRP9: C1q/TNF-related protein 9, EETs: epoxyeicosatrienoic acids, ERK: extracellular signal-regulated kinases, GHRH: growth hormone releasing hormone, GLP-1: glucagon-like-peptide-1, GSK-3 β : glycogen synthase kinase3 beta, HMGB1: high-mobility group protein B1, HSP72: heat shock protein72, IL-6: interleukin-6, JNK: c-jun N-terminal kinase, MAPK: mitogen-activated protein kinase, MEK: methyl ethyl ketone (butanone), NDRG: N-myc downstream-regulated gene, PI3K: phosphatidylinositol kinase, PKC: protein kinase C, PPAR- α : peroxisome proliferator-activated receptor-alpha, RISK: reperfusion injury salvage kinases, RISK: reperfusion injury salvage kinase, ROCK: rho-kinase, sEH: soluble epoxide hydrolase, SERCA: sarco/endoplasmic reticulum Ca²⁺-ATPase, SAFE: survivor activating factor enhancement, tAUBC: trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexylo]-benzoic acid, TIMP-1: Tissue inhibitor of metalloproteinase-1, TMP: 2,3,5,6-tetramethylpyrazine, TNF- α : tumor necrosis factor alpha.

ty. The coronary artery and the biological environment differ significantly to that of experi-

mental models. The presence of concomitant co-morbid conditions, latent preconditioned

Autophagy in ischemia/reperfusion injury

Table 3. Ongoing studies aimed towards limiting reperfusion injury. Note: The trials that cover the cardiac surgical aspects and the maneuvers are excluded

Drug Name of study (NCT)	Sponsor	Target Status
<i>Bendavia</i> EMBRACE (NCT01572909)	Stealth Peptide Inc. Boston, USA	Mitochondrial electron transport chain Ongoing
<i>Cyclosporin A</i> CYCLE (NCT01650662)	Mario Negri Institute for Pharmacological Research, Italy	mPTP inhibitor Ongoing
<i>Cyclosporin A</i> CIRCUS NCT01502774)	Hospices Civils de Lyon, France	Clinical outcomes Ongoing
<i>Nitric Oxide</i> NITRITE-AMI (NCT01584453) NIAMI (NCT01388504) NOMI trial (NCT01398384)	Barts & The London NHS Trust, London, UK	Microvasculatu-re Ongoing
<i>Endovascular catheter cooling</i> CHILL-MI (NCT01379261)	Philips Helthcare, Lund University, Uppsala University	Infarct size reduction Ongoing
<i>Melatonin</i> (NCT01172171)	Herlev Hospital, Aalborg, Denmark	Antioxidant Phase II
<i>TRO40303</i> MITOCARE (NCT001374321)	Trophus, European commission	mPTP antagonist Completed
<i>Atorvastatin</i> The REPERATOR study (NCT00286312)	R&D Cardiologie, Netherlands	Left ventricular remodelling Completed
<i>POST AMI</i> (NCT01004289)	University of Padua, Padua, Italy	Post conditioning Completed
<i>PRIME</i> (NCT01483755)	Hospices Civils de Lyon, France	Delayed post conditioning Completed
<i>Post Cond No Reflow</i> (NCT01208727)	Hospices Civils de Lyon, France	Microvascular obstruction lesions Completed
<i>Streptokinase</i> (NCT00627809)	Istanbul University, Turkey	Microvasculatu-re Completed
<i>Endothelin</i> (NCT00586820)	Mayo Clinic, Minnesota, USA	Microvasculature Completed
<i>Edaravone</i> (NCT00265239)	Kumamoto University, Kumamoto, Japan	Antioxidant Completed
<i>Adenosine</i> (NCT00881686)	Xijing Hospital, Shaanxi, China	Preconditionin-g Completed
<i>FX06</i> , The "F.I.R.E." study (NCT 003269760).	Fibrex Medical Research & Development GmbH,	Fibrin derived peptide B beta 15-42 Completed
<i>Adiponectin</i> <i>R²ACE</i> (NCT01414452)	Universiteit Antwerpen, Antwerp, Belgium	Obesrvational study Completed
<i>Rosiglitazone</i> (NCT00405015)	Radboud University, Nijmegen, Netherlands	Interventional study Completed
<i>Short term Statin</i> (NCT00987974)	Radboud University, Nijmegen, Netherlands	Endothelial dysfunction Completed
<i>Black tea (Camellia sinensis)</i> (NCT01660516)	Radboud University, Nijmegen, Netherlands	Endothelial function Completed
<i>Metformin</i> MetFMD(NCT01610401)	Radboud University, Nijmegen, Netherlands	Endothelial damage Completed
<i>Role of Glucose and Statins</i> (NCT00995670)	Zablocki VA Medical Center, Milwaukee, USA.	Anaesthetic preconditionin-g Completed
<i>Aspirin, Clopidogrel and Statins</i> ACCEL-LOADING-ACS-Study (NCT01354808)	Gyeongsang National University Hospital, Gyeonsangnam-do, Korea	Platelet activation, inflammation and myone- crosis Completed
<i>Remote Post conditioning</i> RemPostCon (NCT00865722)	IRCCS Policlinico S. Matteo, Italy	Post conditioning Completed
<i>Erythropoietin</i> Intra-CO-EpoMI (NCT01043991)	University Hospital, Montmellier,	Infact size reduction Completed

Autophagy in ischemia/reperfusion injury

Milrinone and Esmolol COMET-AMI (NCT 02098629)	Ming-He Huang University of Texas, USA	Infarct size reduction Recruiting
Remote ischemic conditioning CONDI2 (NCT01857414)	Arhus University Hospital Denmark, Serbia, Spain, UK	Clinical outcomes Recruiting
Endothelial arginase inhibitor (NCT02009527)	Karolinska Institute, Stockholm, Sweden	Vasodilation by Nitric Oxide Recruiting
Vit C TREVI (NCT01090895)	University of Soma La Sapienza, Rome, Italy	Antioxidant Recruiting
Spironolactone MINIMISE STEMI (NCT01882179)	University College, London, UK	Infarct size reduction Mineralo-corticoid receptor antagonist Recruiting
Melatonin (NCT0117217)	Herlev Hospital, Aalborg, Denmark	Infarct size reduction Recruiting
Glucagonlike Peptide-1 (NCT02001363)	Chinese PLA General Hospital, Beijing, China	Myocardial salvage Recruiting
DREAM (NCT01664611)	University of Leicester, Leicestershire, UK	Remote ischemic conditioning Recruiting
Cooling STATIM (NCT01777750)	Medical University of Vienna, Austria	Infarct size reduction Recruiting

Source: www.clinicaltrial.gov/CT2/show (accessed on September 10, 2014).

myocardium and the changes associated with medications makes the results variable. Besides, there are inherent complexities and dangers associated with conducting larger human trials which has given rise to numerous experimental studies. We would like to focus on the studies that have been conducted during the last five years.

Studies aimed at suppressing autophagy at the cellular level

Most of the experimental studies that have been conducted are seen to target the PI3K and the Akt pathway to inhibit autophagy. These molecules interact from the cell membranes and have been an easy target to reach. Newer drugs or drugs that have been beneficial have been tried. The final effect is observed as beneficial and cardioprotective or detrimental with elevated levels of autophagosomes, elevated levels of Beclin-1 or the autophagic markers LC3B-II. Besides the aforementioned target, AMPK, Beclin-1 and the paracrine pathways have also been targeted. These studies demand a broader version and translations into human studies. Most of these studies have utilized histological as well as radiological evidence to relate to cardioprotective effect such as decreased in infarct size, improved or good left ventricular functioning. Some of the recently studies autophagic pathways that showed car-

dioprotection with their respective drugs are outlined in **Table 2**.

Results of previous trials

It is worthy to mention here that the clinical studies and in vivo studies are far less in cardiac tissues as compared to non cardiac tissues and animal models. Some of the compounds have gained access to human trials in other diseases like neurodegenerative diseases and cancer. Newer compounds like resveratrol, ginsenoside, Chinese herbal medicine have been impressive in suppressing autophagy in non cardiac tissues and demand further study. Due to the beneficial effects in experimental models, selected drugs have been tried on clinical trials. Some of the important targets have been ROS [151], calcium [152], or pharmaceutical agent that inhibit these or their detrimental effects like-magnesium sulphate [153], sodium-hydrogen exchange inhibitor, adenosine [154], ANP [155], GIK [156], GLP-1 [157], exetanide [158], anti-inflammatory mediators [159-161]. Most of them have demonstrated a positive cardioprotective effects but in variations and have not been able to reach 100% of the set target. The concept of preconditioning and post conditioning in limiting reperfusion injury has been impressive too but it is too early to advocate its application. A clearer, broader role of these pharmaceutical agents and

maneuvers is demanded, which are being searched by wider trials of today.

Future trends

The search for therapeutic target and effective strategy is ongoing and to our dismay, till date, no effective therapeutic strategy exists. Despite these unrewarding searches, newer drugs and their positive interactions continue to be discovered out of these trials. So, human trials are underway, the results of many trials are awaited. Listed in **Table 3** are some newer therapeutic agents and the status of the trials (as the entire exhausting list is not the aim of the article) aimed at restricting reperfusion injury.

Conclusion

Autophagy is integral for cardiac cell survival. Its uncontrolled continuum during reperfusion leads to fatal outcomes which has been the target of many researches. Rigorous search for an effective therapeutic strategy has been unsuccessful so far. A noteworthy point here is manipulating the autophagy pathways and mediators have been impressive in most experimental studies but not suitable for clinical trials due to various reasons. The failed attempts needs to translate into studies centered around the autophagy related genes and autophagy related proteins. Reperfusion injury cannot be attended without taking care of autophagy process as reperfusion of a cardiac tissue cures the MI like the warm tip of an iceberg; the submerged injuries are vast, rigid and detrimental. However the concern that this process has received is indeed encouraging and we hope that the results of many molecular targets, which are awaited, initiated or planned would surely relate to effective cardio protection in the future.

Disclosure of conflict of interest

None.

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