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Autophagy in Ischemic Livers: A Critical Role of Sirtuin 1/Mitofusin 2 Axis in Autophagy Induction

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No-flow ischemia occurs during cardiac arrest, hemorrhagic shock, liver resection and transplantation. Recovery of blood flow and normal physiological pH, however, irreversibly injures the liver and other tissues. Although the liver has the powerful machinery for mitochondrial quality control, a process called mitophagy, mitochondrial dysfunction and subsequent cell death occur after reperfusion. Growing evidence indicates that reperfusion impairs mitophagy, leading to mitochondrial dysfunction, defective oxidative phosphorylation, accumulation of toxic metabolites, energy loss and ultimately cell death. The importance of acetylation/deacetylation cycle in the mitochondria and mitophagy has recently gained attention. Emerging data suggest that sirtuins, enzymes deacetylating a variety of target proteins in cellular metabolism, survival and longevity, may also act as an autophagy modulator. This review highlights recent advances of our understanding of a mechanistic correlation between sirtuin 1, mitophagy and ischemic liver injury.

Key words: Autophagy, Mitochondria, Liver, Ischemia/Reperfusion, Acetylation

INTRODUCTION

Timely removal of unnecessary cellular constituents and abnormal organelles is essential to sustain cell viability. Intracellular protein degradation and protein synthesis are tightly balanced to maintain cell survival. Two catabolic pathways account for protein degradation: 1) the ubiquitin-proteasome pathway for degradation of short-lived proteins, and 2) the autophagy or "self-eating" pathway for degradation of long-lived proteins and abnormal organelles (1). In the liver, long-lived proteins constitute more than 99% of cell proteins and thus, autophagic degradation is the primary catabolic process for proteins (2). Three major forms of autophagy have been described in mammalian cells: microautophagy, macroautophagy and chaperone-mediated autophagy (1). Among the three forms, macroautophagy is of particular importance in the liver as this form of autophagy not only clears unneeded intracellular proteins but also digests injured or dysfunctional organelles such as abnormal mitochondria. Although macroautophagy is generally considered a random process, growing evidence shows the existence of selective macroautophagy, especially for clearance of the mitochondria, termed mitophagy (3).

The liver is vulnerable to hypoxic and anoxic stresses. Although prolonged interruption of blood flow (ischemia) eventually causes hepatocyte death, extensive injury paradoxically occurs mostly after the restoration of blood flow and oxygen, a phenomenon called reperfusion injury (4). Ischemia/reperfusion (I/R) injury is a major pathological event in low flow disease states, including hemorrhagic shock, and cardiac arrest, and intentionally during surgical procedures such as liver resection and transplantation. Mitochondrial dysfunction is a causal mechanism attributing to I/R injury (4,5). Injured mitochondria hamper energy production and precipitate further injury to neighboring mitochondria through release of cytotoxic compounds (5). Therefore, elimination of damaged mitochondria in a timely manner is critical to sustain viability in ischemic hepatocytes. As mitophagy is the only known cellular mechanism to dispose abnormal mitochondria, active recruitment of mitophagy has a therapeutic potential for mitochondria-related diseases (4).

THE LIVER

The liver, the largest internal organ located in the upper right quadrant of the abdomen, performs multiple functions

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in the body, including synthesis of essential proteins and cofactors; regulation of glycogen synthesis and degradation; storage of vitamins and minerals; production and secretion of hormones and bile; and clearance of toxic metabolites. To accomplish these varied functions, distinct types of hepatic cells - hepatocytes, Kupffer cells, stellate cells, sinusoidal endothelial cells, cholangiocytes, lymphocytes and dendrite cells - require high amounts of cellular energy in order to synthesize and eliminate a plethora of complex molecules. Such a high demand for cellular energy in the liver can likewise be inferred from its unique dual-blood supply system: 1) the portal venous supply from the gut, pancreas, and spleen, and 2) arterial supply from the heart (6). Using these two sources of blood supply, the liver is continuously nourished with oxygen, energy substrates and nutrients. Hepatic blood vessels encompass about 22% of the liver mass/volume and the liver contains about 12% of the total blood volume (7). Blood flow through the liver amounts to 1,500~2,000 mL/min (8). The hepatic artery and the portal vein furnish approximately 25% and 75% of the resting cardiac output, respectively, and complete mixture of both portal and arterial blood occurs in the hepatic sinusoids (6). Hence, the liver is a highly aerobic organ and innately vulnerable to hypoxic and ischemic stresses. Sinusoidal blood flow to the hepatic lobules and subsequent addition of synthetic products and metabolic wastes to the blood create gradients of oxygen and metabolites between periportal and pericentral regions of the liver lobule (9). As a consequence, the liver exhibits a distinct zonal dependence on specific biochemical reactions. For instance, while ureagenesis, gluconeogenesis, beta-oxidation of fatty acids, and cholesterol synthesis are enriched in the periportal hepatocytes, lipogenesis, glycolysis and drug detoxification occurs mainly in the pericentral hepatocytes (9). Hepatic disease likewise demonstrates zonal dependency. Hypoxic injury is observed first in the pericentral region due to the intralobular oxygen gradient (10). Hemosiderin accumulation in hemochromatosis and drug-induced hepatotoxicity frequently exist in periportal and pericentral area, respectively (7).

LIVER SURGERY AND I/R INJURY

Chronic liver disease is the fifth leading cause of death worldwide (11). Total deaths worldwide from liver cirrhosis and cancer have increased from 676,000 in 1980 to over 1 million in 2010 (12). Liver resection refers to the surgical removal of portions of the liver that contain cancer, benign tumors or cystic disease. A common technique employed in liver resection in order to reduce intraoperative blood loss is the Pringle maneuver, which involves the clamping of the portal triad (hepatic artery, portal vein, and bile duct) (6). When this technique is employed, the blood flow to the liver is interrupted, resulting in oxygen and nutrient depletion and subsequently impeding mitochondrial oxidative phosphorylation and ATP generation (4). At the same time, stored glycogen hydrolyzes into glucose that prompts anaerobic glycolysis (13). Although this cytosolic glucose utilization pathway replenishes some ATP to the liver, the end product, lactic acid, accumulates in the cell resulting in tissue acidosis. Moreover, hydrogen ions released from ATP hydrolysis and acidic organelles during ischemia further enhance tissue acidosis (14,15). Restoration of blood flow upon release of vascular clamping results in a return to physiologic pH, an event that worsens ischemic tissue injury (4,16). Another setting from which I/R injury can occur to the liver is liver transplantation. Approximately 6,700 liver transplantations are performed annually in the United States (17). Donor livers exposed to ischemia during harvest, storage and transport undergo subsequent reperfusion injury once they are anastomosed to recipient vasculature, and blood flow is restored.

MECHANISMS OF I/R INJURY

The mechanisms underlying hepatic I/R injury are multifactorial and include Ca^{2+} deregulation, mitochondrial dysfunction, generation of reactive oxygen and nitrogen species, loss of cellular antioxidants, stimulation of catabolic enzymes, and loss of autophagy (4). Progression of I/R injury can be viewed as three different stages: Within the first few minutes of reperfusion, sequential events of calcium overloading and reactive oxygen species (ROS) accumulation in the mitochondria cause mitochondrial dysfunction and the onset of mitochondrial permeability transition (MPT), leading to ATP depletion and necrotic death of hepatocytes. (4,5) Increased ROS also activate Kupffer cells to promote even greater ROS production. During the next 6 hrs of reperfusion, activated Kupffer cells continue to release ROS, cytokines and chemokines to recruit neutrophils. Finally, at the late phase of reperfusion, neutrophil infiltration becomes uncontrollable and incites irreversible systemic inflammation. Although tissue inflammation is an important pathology in hepatic I/R injury, it should be noted that the damage to hepatocytes after reperfusion is the earliest event that conduces to permanent I/R injury to the liver (4,5).

Mitochondrial dysfunction is the main causative mechanism of hepatocyte death after I/R (4,16). The mitochondrion, a power plant in the cell, contains both inner and outer membranes. In contrast to the mitochondrial outer membrane, the mitochondrial inner membrane is virtually impermeable to all solutes except for those having specific carriers or exchangers. While acidic pH during ischemia prevents mitochondrial permeabilization, the opening of MPT pores upon reperfusion disrupts the inner membrane barrier in the mitochondria, which allows an unregulated influx of solutes up to 1,500 Da into the mitochondrial matrix (5,16). Free diffusion of solutes successively induces mitochondrial

swelling, uncoupling of oxidative phosphorylation, and depolarization of the mitochondrial membrane potential. As the proton motive force in the mitochondria is governed equally by mitochondrial membrane potential and pH gradient, the onset of MPT after reperfusion collapses the proton motive force, causing ATP depletion and cellular necrosis. Thus, MPT onset and subsequent mitochondrial dysfunction are the key mechanisms contributing to hepatocyte death after I/R (5,16). Besides necrosis, the onset of MPT can also provoke apoptotic cell death. The loss of mitochondrial membrane integrity releases cytochrome *c* that is normally sequestered in the intermembrane space in the mitochondria. Once released, cytochrome *c* binds to the apoptosis-inducing factor-1 and pro-caspases to form a protein complex, the apoptosome, which, in turn, activates downstream effector caspases and develops apoptosis (16,18). Thus, MPT onset is a common pathway to both necrotic and apoptotic hepatocyte death after reperfusion. In contrast to necrosis, apoptosis requires ATP. The availability of glycolytic ATP is a critical determinant of cell death fate (18): When hepatocytes are depleted of ATP after MPT onset, necrosis is a predominant cell death fate. However, when ATP is available, hepatocytes undergo apoptotic death instead (16).

Molecular composition of the MPT pores remains incompletely understood; however, three major components, the adenine nucleotide translocator (ANT) on the inner mitochondrial membrane, voltage-dependent anion channel (VDAC) on the outer mitochondrial membrane, and cyclophilin D in the mitochondrial matrix have been identified (16,19). Multiple studies have proposed that the conformational change from *trans* to *cis* ANT by cyclophilin D results in MPT onset (16,19-21). Other proteins might also play a role, including hexokinase and Bcl-2 family members (16). Low pH and cyclosporine A (CsA), an immunosuppressive agent, suppress the MPT by inhibiting cyclophilin D (21). While the permanent opening of MPT pores inevitably accompanies cell death, transitory opening plays an integral role for sustaining normal cellular physiology. A transient cycle of mitochondrial pore opening and closing is necessary in Ca^{2+} and ROS-mediated signal transduction, while concomitantly serving protective functions against unexpected overloading of intramitochondrial Ca^{2+} and ROS (20).

Therapeutic strategies to prevent hepatic I/R injury have included antioxidant therapy, steroids, Ca^{2+} chelators, MPT blockers, and protease inhibitors but the clinical outcomes from these therapies to date remain disappointing (22-26). For instance, MPT blockers like CsA, N-methylvaline cyclosporin, and N-methyl-4-isolleucine cyclosporin (NIM811) have a narrow range of therapeutic efficacy (16,27,28) and CsA-insensitive MPT can also develop when MPT induction increases (21). It should also be noted that mitochondrial overloading of Ca^{2+} and overproduction of ROS, events upstream to the MPT, still prevail even after the MPT is blocked (10). Therefore, it is unlikely that significant and persistent levels of cytoprotection could be achieved with current therapeutic strategies. As mitophagy selectively targets and removes abnormal mitochondria, this endogenous mitochondrial quality control machinery could have therapeutic potentials for I/R injury and other mitochondrial diseases.

AUTOPHAGY

Autophagy is an evolutionarily conserved catabolic process that eliminates protein aggregates and surplus or damaged intracellular organelles. Autophagy was first described by Christian de Duve and is defined as a cell's "self-eating" event (29). As macroautophagy clears both cellular constituents and dysfunctional mitochondria, this review focuses on macroautophagy and refers it to as autophagy hereafter.

Autophagy is a sequential process that begins with the initiation and formation of an autophagosome, a double membrane structure that sequesters and delivers cellular cargo to the lysosome. Canonical autophagy relies on the recruitment of multiple autophagy-related proteins (ATG) onto a cup-shaped double membrane complex termed a phagophore. Non-canonical autophagy is incompletely understood but can occur in the absence of some key ATG proteins where the expansion of phagophore membrane is dependent on vesicular transport vesicles originated from the Golgi and endosomes (30). Autophagy can selectively or non-selectively enclose cargo material. Selective autophagy includes the removal of specific cellular constituents and intracellular organelles: peroxisomes (pexophagy) (31), mitochondria (mitophagy) (3), ribosomes (ribophagy) (32), endoplasmic reticulum (reticulophagy) (33), lipids (lipophagy) (34), and iron (ferrintinophagy) (35).

Autophagy is slow under basal conditions but becomes stimulated by certain conditions such as nutrient depletion or starvation (1,29). Under nutrient and amino acid-rich conditions, UNC-51 like kinase 1 (ULK1), a mammalian ortholog of yeast ATG1, is phosphorylated at the residue of Ser757 by mammalian or mechanistic target of rapamycin complex 1 (mTORC1) and dissociated from adenosine monophosphate-activated protein kinase (AMPK). This process promotes cell growth and proliferation but prevents autophagy initiation (36). However, under nutrient insufficiency, AMPK senses the changes in energy contents and initiates autophagy by phosphorylating the residues of Ser317, 555, 777 of ULK1 (37,38). Activated ULK1 serves in recruiting and phosphorylating both ATG13 and focal adhesion kinase family interacting protein of 200 kDa (FIP200), generating the ULK1-ATG13-FIP200 complex on the surface of phagophores (39,40). Moreover, ULK1 subdues the kinase activity of mTORC1 through its binding with raptor (41). Thus, the orchestrated coordination between mTORC1, AMPK, and ULK1 is an integral part of autophagy initiation. Another important event in autophagy initiation is the phosphorylation and activation of Beclin -1 (BECN1). When

nutrients are abundant, anti-apoptotic protein, B cell lymphoma-2 (Bcl-2), binds to BECN1 and inhibits autophagy. In contrast, under nutrient insufficiency, Bcl-2 is phosphorylated by stress-responsive c-Jun N-terminal protein kinase 1 (JNK1). The change in phosphorylation status of Bcl-2 dissociates BECN1 from Bcl-2, and liberated BECN1 then stimulates autophagy (42,43). BECN1 is also a key component in the BECN1-ATG14-VPS34-VPS15 class III PI3K core complexes (44). Vacuolar sorting protein 34 (VPS34), a class III lipid kinase, modulates vesicle trafficking and the formation of autophagosomal membranes (45,46). The initiation of autophagy is further regulated by other factors, including ultraviolet irradiation resistance-associated gene (UVRAG) (47), BIF-1 (48), ATG14L (49) or RUN domain Beclin-1-interacting cysteine-rich-containing protein (RUBICON) (50).

ATG12 and microtubule-associated protein 1 light chain 3 (LC3) play a major role in autophagosome maturation. Upon the initial activation by ATG7, an E1-ubiquitin like enzyme, ATG12 is covalently connected to ATG5 by ATG10, an E2-like ubiquitin carrier protein. Conjugated ATG12-ATG5 complexes then interact with Atg16L1 later. Other proteins including ATG4B, ATG7, ATG3, are also involved in the maturation of autophagosomes (51). The conjugation of LC3-I to phosphatidylethanolamine by ATG7 and ATG3 generates LC3-II that localizes to autophagosomal membranes. On the contrary, unconjugated LC3-I resides in the cytosol. Due to its distinct location and unique chemical structure, LC3-II is commonly used to monitor autophagy (52,53). The conversion of LC3-I to LC3-II requires ATG4B, a cysteine protease. Importantly, ATG4B can also act on LC3-II to release LC3 from phosphatidylethanolamine (54). The removal of phospholipid not only relocates LC3 from the autophagosomal membrane, but also facilitates its subsequent fusion with the endosome/lysosome.

The mature autophagosome fuses with the lysosome to produce the autolysosome. It has been shown that Ras-like GTPases are involved in this tethering process (55). Specifically, overexpression of Rab7 promotes autophagy, whereas its silencing prevents autophagy (56). The interaction of UVRAG-BECN1-PI3KIII complex with the class C vacuolar protein sorting complex further facilitates Rab7-mediated fusion (57). The formation of autolysosomes is also fine-tuned by soluble N-ethylmaleimide-sensitive fusion factors (SNARES) (57,58), showing the complexity of autophagy network. Once the formation of autolysosomes is completed, its luminal contents are rapidly degraded by acidic proteases, lipases, nucleases, and glycosidases. The final end products following this process are later recycled back to the cell for other metabolic purposes (37).

MITOPHAGY

Mitophagy mediates mitochondrial turnover, which occurs every 15 to 25 days (59). Therefore, functional mitophagy not only prevents the accumulation of abnormal or damaged mitochondria, but also is essential to maintain a stable number of healthy mitochondria. Lemasters' group has proposed that three different types of mitophagy exist in the cell (3). The mechanisms of type I mitophagy are similar to those in canonical autophagy described above. This mitophagy requires PI3KIII signaling and can occur at the phagophore assembly. In contrast, mitochondrial depolarization instigates the onset of type II mitophagy. Type III mitophagy, termed "micromitophagy," depends on the formation of mitochondria-derived vesicles enriched in oxidized mitochondrial proteins that bud off and transit into multivesicular bodies. Overall, both type I and II mitophagy engulf an entire mitochondrion for removal, while type III selectively eliminates damaged and oxidized mitochondrial components.

Several proteins have been proposed to induce mitophagy. Under normal conditions, the mitochondrial serine protease, presenilin-associated rhomboid like protein (PARL) cleaves tension homolog-induced putative kinase protein 1 (PINK1) in the mitochondria (60). When the mitochondria depolarize under stress conditions, a decrease in PARL activity and following inhibition of PINK cleavage translocate a full length PINK1 to the outer mitochondrial membrane. Soon after, PINK1 recruits PARKIN, an E3 ubiquitin ligase, to the mitochondria where PARKIN directs the ubiquitination of target proteins such as p62 and VDAC of damaged mitochondria (61-63). Transcription factor p62 is known to act as a linker protein between autophagic cargo and autophagosomes (64). The mitochondrial accumulation of PARKIN appears to be voltage-dependent, and does not require changes in pH or ATP levels (65). Mitochondrial receptors Bcl2/adenovirus EB 19-kDA interacting protein 3 (BNIP3) or FUN14 domain-containing protein-1 (FUNDC1) likely also plays a role in mitophagy (66). BNIP3, also called BNIP3L or NIX, shares homology with Bcl-2 in the BH3 domains. FUNDC1-mediated mitophagy requires ULK1, wherein activated ULK1 phosphorylates FUNDC1 upon mitochondrial depolarization (67,68). Multiple studies have posited that BNIP3 and FUNDC1 trigger mitophagy by binding to LC3 through a WXXL motif (66,69,70).

MITOPHAGY IN LIVER I/R INJURY

Autophagy is a highly energy-dependent process. Hence, ATP depletion during hepatic I/R adversely impacts the autophagic machinery. Anoxia during ischemia impedes the formation of autophagic vesicles, as evidenced by lack of LC3-II increase in the presence of lysosomal inhibitors such as bafilomycin or chloroquine (71,72). Although a transient repolarization of the mitochondrial membrane potential during the early stage of reperfusion can provide some ATP to cells and operate autophagy temporarily, the demand for mitoph-

agy to remove swollen and injured mitochondria exceeds the autophagic capacity in reperfused hepatocytes. A few minutes after reperfusion, hepatocytes thus encounter accumulation of abnormal or dysfunctional mitochondria, uncontrolled Ca^{2+} and ROS overloading, activation of injurious enzymes, the onset of MPT and eventually cell death (5,71,72). To make things worse, key autophagy proteins such as ATG7 and BECN1 become hydrolyzed by calpains as a consequence of Ca^{2+} overloading (5,71,72). Hence, loss of key autophagy proteins and depletion of ATP synergistically impair mitophagy after I/R. The importance of mitophagy in ischemic livers is substantiated by findings that both pharmacological and genetic approaches that stimulate mitophagy confer cytoprotection against hepatic I/R injury (5,71,72). Of note, observations that initial MPT onset occurs in a subset of mitochondria prior to widespread MPT in the cell suggest that some mitochondria are more prone to I/R stress. Toxic metabolites and byproducts from injured mitochondria can propagate to neighboring healthy mitochondria, culminating in widespread mitochondrial dysfunction (73). Since mitophagy enhancement blocks the onset of MPT and cell death after reperfusion, timely clearance of these stress-prone mitochondria appears to be indispensable for sustaining functional bioenergetics and cell survival.

SIRTUINS IN THE LIVER

Acetylation is a post-translational modification of proteins by covalent addition of an acetyl group to lysine residues. In general, removal of positively-charged lysine neutralizes the total charge balance of target proteins and thus alters the steric environment of their active sites. Acetylation or deacetylation can impact a variety of cellular functions such as DNA binding affinity, catalytic activity, stability and localization of target proteins (74). Protein acetylation is a highly dynamic process that is governed by balanced action between lysine acetyltransferases (KATs, formerly known as histone acetyltransferases, HATs) and deacetylases (KDACs, formerly termed as histone deacetylases, HDACs). KATs are categorized into three major groups: 1) KAT2/GCN5-related N-acetyltransferases (GNAT family), 2) E1A binding protein p300 (EP300/CREBBP family), and 3) MYST family (75). KDACs are further subdivided into 4 classes, based on their sequence homology to the original yeast enzymes and domain organization. Designated as Class III KDACs, sirtuins have some distinctive features from other classes. They are mammalian ortholog of yeast silent information regulator 2 (Sir2) and utilize oxidized nicotinamide adenine dinucleotide (NAD^+) as a cofactor for their enzyme activity (76-80). In mammals, seven different isoforms of sirtuins (SIRT1-7) have been identified. Although individual isoforms contain a uniquely conserved NAD^+ deacetylase domain, deacetylation activity varies among the isoforms. The conserved catalytic domain of sirtuins contains up to 270 amino acid residues and forms a characteristic reverse Rossmann-fold, and zinc ribbon (81,82). Recent reports that acetyl-coenzyme A (AcCoA), a major component of the Krebs cycle and β-oxidation in the mitochondria as well as glycolysis and catabolism of branched amino acids in the cytosol, donates its acetyl moiety to the target lysine residues (83,84) demonstrate the involvement of acetylation reactions in cellular energy metabolism. Expectedly therefore, studies confirm that deacetylation reactions by sirtuins are distinctly coupled to transcription, mitochondrial biogenesis, oxidative phosphorylation, and autophagy (85,86).

Sirtuin 1 (SIRT1) localizes in the cytosol and nucleus (87) and is known to regulate circadian rhythms (88-90), autophagy (91-95), gluconeogenesis (96,97), fatty acid oxidation (96,98), mitochondrial biogenesis (96,99,100), cell proliferation (101,102) and antioxidant defense (92). Embryonic lethality in SIRT1-null transgenic mice implicates its essential role in tissue viability (103). In the liver, SIRT1 can deacetylate a myriad of non-histone targets including peroxisome proliferator-activated receptor gamma-coactivator-1 alpha (PGC-1α) (97), CREB regulated transcription coactivator 2 (104), Forkhead transcription factors (FOXO) (105), fibroblast growth factor 21 (FGF21) (106), and signal transducer and activator of transcription 3 (STAT3) (107), all of whch are closedly associated with hepatic energy homeostatsis and metabolism.

The roles of other sirtuin isoforms in physiology and pathophysiology are beginning to be elucidated as well. The mitochondria-localized SIRT3, for instance, modulates intramitochondrial metabolic activities and ROS formation by deacetylating the NDUFA9 subunit of Complex 1 in the mitochondrial electron transfer chain (108-112). Another connection of SIRT3 with mitochondrial energy homeostasis comes from experiments demonstrating that SIRT3 can directly deacetylate mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2, a late limiting enzyme in the synthesis of ketone body (112-114). SIRT5 is another mitochondrial isoform of sirtuins encompassing deacetylation (115), desuccinylation (116), demalonylation (116), and deglutarylation (117). This mitochondrial matrix enzyme has long been known to regulate carbamoyl phosphate synthase 1 (CPS1), a rate limiting enzyme for the urea cycle and ammonia clearance (115-117). Taken together, both mitochondrial and extramitochondrial sirtuins affect cellular energy metabolism and homeostasis.

ROLE OF ACETYLATION/DEACETYLATION IN AUTOPHAGY

Although sirtuins are not an essential component of the autophagic machinery, evidence is accumulating to indicate that autophagy is regulated by the cycle of acetylation/deacetylation. Table 1 summarizes autophagy-related pro-

Table 1. Summary of autophagy related proteins regulated by acetylation/deacetylation

Protein	Description	References
ULK1	Enhancing autophagy upon acetylation by TIP60	(123,124)
ATG3, ATG5, ATG7	Decreasing autophagy upon acetylation by p300 Enhancing autophagy upon deacetylation by SIRT1	(91,120)
ATG12	Decreasing autophagy upon acetylation by p300	(120)
LC3	Decreasing autophagy upon acetylation by p300 Enhancing autophagy upon deacetylation by SIRT1	(91,120,121)
BECN1	Decreasing autophagy upon acetylation by p300 Enhancing autophagy upon deacetylation by SIRT1	(122)
FOXO1, FOXO3	Decreasing autophagy upon acetylation by p300 Enhancing autophagy upon deacetylation by sirtuins (SIRT1, 2 and 3)	(119)
MFN2	Enhancing autophagy upon deacetylation by SIRT1	(125)
Tubulin	Enhancing autophagy upon acetylation by α-TAT1/MEC17 Decreasing autophagy upon deacetylation by HDAC6	(128,129)
Hsp70	Decreasing autophagy upon acetylation by p300 Enhancing autophagy upon deacetylation by HDAC6	(130)

tein targets that are regulated by acetylation status. Acetylation/deacetylation-dependent modulation of autophagy occurs transcriptionally and translationally. For example, spermidine, a natural inhibitor of KATs, enhances autophagy through hypoacetylation of ATG7 promoter (118). Studies have shown that changes in acetylation status significantly impact the activity of FOXO1 or FOXO3, transcription factors associated with autophagy induction (119). More direct evidences come from the studies which demonstrate that p300 can acetylate ATG5, ATG7, LC3 and ATG12 (120), whereas SIRT1 can deacetylate ATG5, ATG7 and LC3 under nutrient insufficiency (91). SIRT1-dependent deacetylation also determines intracellular distribution of autophagy components. Huang et al. recently reported that deacetylation of LC3 by SIRT1 redistributes LC3 from the nucleus to the cytoplasm, and that deacetylated cytosolic LC3 produces more stable autophagosomes (121). Thus, acetylation/deacetylation cycle not only regulates the activity of autophagy but also ensures an effective redistribution of autophagy elements between intracellular compartments. BECN1 appears to be another autophagy target that is regulated by acetylation/deacetylation since acetylated BECN1 inhibits autophagosome maturation and endocytic trafficking (122). Studies also suggest that acetylated ULK1 can enhance autophagy process by stimulating its kinase activity (123,124). Thus, changes in acetylation status highly affect both initiation and elongation stage of autophagy.

SIRT1, AUTOPHAGY AND I/R INJURY

We recently demonstrated in human liver biopsies that hepatic inflow occlusion during liver resection decreases SIRT1 expression to 30% of basal levels (125). Such a reduction was also evident in the mouse livers and hepatocytes after *in vivo* and *in vitro* I/R, respectively. Calpain activation due to Ca^{2+} overloading during I/R appears to be, at least in part, responsible for SIRT1 loss. Although reperfusion after prolonged ischemia leads to near-complete depletion of SIRT1 in both the cytosol and nucleus, subcellular fractionation assays revealed that cytosolic SIRT1 loss precedes nuclear SIRT1 loss, implying that cytosolic SIRT1 is more susceptible to hepatocellular I/R injury. Adenoviral overexpression or pharmacological activation of SIRT1 by resveratrol or SRT1720 enhanced cytosolic levels of SIRT1 and mitophagy, and sustained mitochondrial structural integrity after reperfusion, substantiating the importance of cytosolic SIRT1 in mitochondrial quality. The mechanism by which SIRT1 elevates mitophagy is likely associated with ATG7 because SIRT1 overexpression significantly increases ATG7 expression, whereas levels of other autophagy-related proteins such as ATG3, ATG4B, ATG12-5, ATG14L, BECN1, RUBICON, LAMP2A, and Cathepsin D remain unaltered by this treatment. Importantly, both treatments conferred cytoprotection against global MPT onset and necrosis, which was not observed in hepatocytes from SIRT1 conditional knockout mice. In contrast to wild-type hepatocytes, the cells from SIRT1-deficient mice exhibited a rapid onset of the MPT and increased cell death even after short ischemia. Taken together, these results indicate that SIRT1 depletion contributes to I/R injury in hepatocytes and cytosolic SIRT1 is required for mitochondrial integrity and function (Fig. 1).

Many mitochondrial proteins exist in an acetylated form. About 35% of all mitochondrial proteins are endogenously acetylated, 24% of which are mechanistically linked to energy homeostasis (126). Hyperacetylation or defective

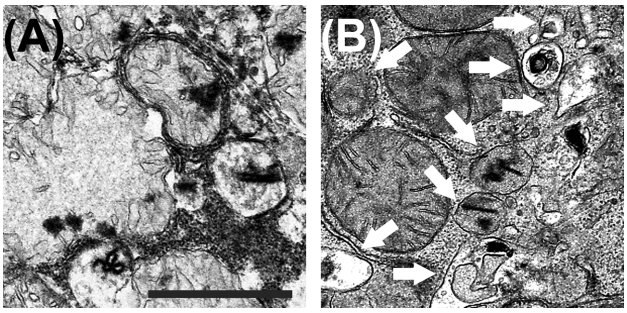

Fig. 1. The effects of sirtuin 1 on the mitochondrial integrity and autophagy in reperfused hepatocytes. (A) Scanning electron microgram shows that in the control cell, reperfusion of ischemic hepatocytes causes a marked swelling and structural disruption of the mitochondria. Note lack of autophagy induction under this condition. (B) However, overexpression of sirtuin 1 prior to I/R sustains the integrity of mitochondrial structure. Under this condition, numerous autophagic vesicles are visible (arrows). Scale bar: 2 μm.

deacetylation of the mitochondrial proteins has been shown to account for liver steatosis and obesity (114,127). The mechanisms underlying SIRT1-mediated cytoprotection against I/R injury are likely linked to deacetylation of mitofusin 2 (MFN2), a mitochondrial outer membrane protein, by SIRT1 and subsequent augmentation of mitophagy. Immunoprecipitation and immunoblotting approaches unveiled that while cytosolic SIRT1 physically interacts with both MFN1 and MFN2, it deacetylates only MFN2. The importance of SIRT1/MFN2 interaction in hepatic I/R injury was further supported by the result that knock-down of MFN2 with a small hairpin RNA abolishes a series of beneficial effects by SIRT1, including SIRT1-mediated mitophagy induction, cytoprotection against mitochondrial dysfunction, and cell death after reperfusion. Though the acetylated residues of mouse MFN2 are currently unknown, bioinformatic analysis conforming to either X6-K-[Y,W,F]-X5 or X6-KX5-[Y,W,F] motif predicts at least five different SIRT1 target sites of MFN2: K37, K215, K357, K655, and K662 (125). Noticeably, K215 localizes at the GTPase domain, a critical site for catalytic activity of MFN2. Both K655 and K662 reside in the C-terminal flanking domain that directs mitochondrial localization of MFN2. Consistent with this prediction, deletion of N-terminal regions of MFN2 blunted SIRT1-mediated autophagy induction. One interesting observation is that while hepatocytes are relatively tolerant of a large loss in SIRT1, a similar reduction of MFN2 causes greater cell death after reperfusion, implying a central role of MFN2 in I/R injury to the liver. It has been reported that MFN2-null animals are embryonically lethal, whereas SIRT1 knockout mice are born alive (125). Hence, it is speculated that MFN2-deficient cells may be more prone to I/R injury than SIRT1-null counterpart. Although the minimal levels of MFN2 needed for adequate responses to I/R and other

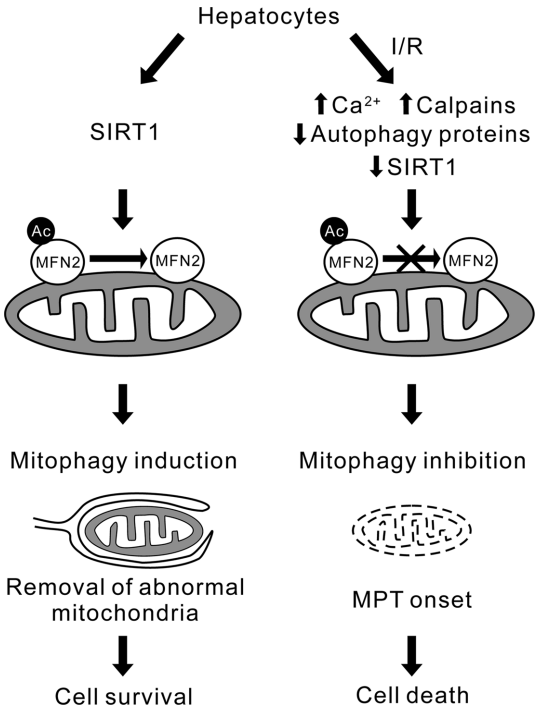

Fig. 2. Proposed mechanism of sirtuin 1-mediated cytoprotection against ischemia/reperfusion injury in the liver. In normal hepatocytes, cytosolic sirtuin 1 (SIRT1) deacetylates mitofusin 2 (MFN2), a mitochondrial outer membrane protein, which, in turn, induces mitophagy and maintains a stable number of heathy mitochondria and cell viability thereafter. In sharp contrast, ischemia/reperfusion (I/R) to hepatocytes causes Ca^{2+} overloading and calpain activation that subsequently hydrolyzes both key autophagy proteins and SIRT1. As a consequence, MFN2 remains acetylated (Ac-MFN2) and the onset of mitophagy fails. Hepatocytes further accumulate intramitochondrial Ca^{2+} and reactive oxygen species, resulting in the mitochondrial permeability transition (MPT) and cell death.

stresses remain to be determined, the interaction of MFN2 and its subsequent deacetylation by SIRT1 are likely pivotal events in autophagy regulation and cell survival after I/R (Fig. 2).

CONCLUSION AND FUTURE PERSPECTIVES

I/R injury has a profound impact on the burden of liver diseases. Efforts to improve liver function after I/R, however, have not been successful largely due to an incomplete understanding of I/R injury. The mechanisms behind hepatic I/R injury are multifactorial, including defective mitophagy, the onset of MPT and mitochondrial dysfunction. Such a complexity of reperfusion injury is further underscored by the recent study which shows that the SIRT1/MFN2 axis controls mitophagy and mitochondrial function in ischemic livers. Although enhancing mitophagy has emerged as a new potential strategy against reperfusion injury, there exist

a few unanswered questions, such as the effects of mitophagy on non-parenchymal cells after I/R, roles of different types of mitophagy in ischemic livers, similarities and differences in mitophagy signaling pathways between normal and ischemic livers, and impact of other mitochondrial sirtuins and their potential interactions with SIRT1 before and after ischemia. It should also be noted that current strategies including treatment with autophagy inducers prior to ischemia and the viral delivery of specific autophagy genes and lack of specific autophagy enhancers without compromising the immune system all limit their clinical applications. Better understanding of the pathological complexity of reperfusion injury and its mechanistic insights into mitophagy could lead to the development of promising treatment strategies for hepatic I/R injury and mitochondrial diseases.

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