MITOFUSIN 2 MEDIATES SIRTUIN 1 INDUCED AUTOPHAGY TO SUPPRESS LIVER ISCHEMIA/REPERFUSION INJURY

By

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To my mom and dad for their support, patience and love through my many endeavors
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MFN2 mediates SIRT1 induced autophagy to suppress against liver I/R injury.
Hepatic ischemia/reperfusion (I/R) injury causes organ damage that can lead to liver failure and mortality after resection and transplantation surgeries. Currently, there are no therapeutic approaches to circumvent liver I/R injury. Autophagy is a lysosomal dependent catabolic process that degrades long-lived proteins and dysfunctional organelles. Autophagy is an endogenous cytoprotective mechanism to suppress liver I/R injury. Sirtuin 1 (SIRT1) is a NAD^+ dependent deacetylase that mediates autophagy, thus we investigated the role of SIRT1 during liver I/R injury. Human and mouse livers, and primary hepatocytes were subjected to I/R conditions to determine changes in SIRT1 expression. A dramatic reduction in SIRT1 was observed in both livers and hepatocytes subjected to I/R, which was partly dependent on both cathepsins and calpains. Modulation of SIRT1 using an adenovirus expressing SIRT1, or pharmacological activators, Resveratrol and SRT1720, suppressed liver I/R injury, while the loss of SIRT1 sensitized hepatocytes to I/R. Activation of SIRT1 enhanced autophagy before and after I/R, which was absent in SIRT1 deficient hepatocytes suggesting that SIRT1 induced autophagy suppresses liver I/R injury. To determine the
mechanisms of SIRT1 induced autophagy, we analyzed autophagy related protein expression, autophagy initiation signals, potential protein interactions and substrate acetylation. Mitofusin 1 (MFN1) and Mitofusin 2 (MFN2) were identified as acetylated proteins that form a complex with SIRT1. Furthermore, overexpression SIRT1 led to MFN2 deacetylation. Next, we demonstrated that SIRT1 induced autophagy was impaired in MFN2 deficient hepatocytes prior to and after I/R. To validate the importance of MFN2 during I/R, livers and hepatocytes were subjected to I/R, which caused a significant MFN2 reduction in a manner partly dependent on cathepsins and calpains leading to hepatocyte death. Overexpression of SIRT1 suppressed the loss of MFN2 and enhanced autophagy flux during I/R, while MFN2 knockdown hepatocytes had an impaired autophagic flux and were hypersensitive I/R. Collectively, these data suggest that MFN2 mediates SIRT1 induced autophagy to suppress liver I/R injury.
CHAPTER 1
AUTOPHAGY SUPPRESSES LIVER ISCHEMIA/REPERFUSION INJURY AND THE THERAPEUTIC POTENTIAL FOR TARGETING SIRTUINS

Introduction

Ischemia/reperfusion (I/R) injury is a primary factor that causes liver failure and patient mortality after liver resection and transplantation surgeries. Therapeutic advances against warm I/R injury have been disappointing, thus new strategies are required. Mitochondrial dysfunction is the causative mechanism leading to hepatocyte death and liver failure after warm I/R. Upon reperfusion, dysfunctional mitochondria accumulate leading to hepatocyte death and liver damage. Autophagy is an endogenous mechanism that can degrade dysfunctional mitochondria, however I/R impairs autophagy. Activation of autophagy during I/R leads to the removal of dysfunctional mitochondria to protect against liver damage. Sirtuins are class III histone deacetylases that are dependent on nicotinamide adenine dinucleotide (NAD\(^+\)). There are seven sirtuin isoforms that localizes to different cellular compartments. Activation of Sirtuin 1 (SIRT1) enhances autophagy and mitochondrial biogenesis, however the role of SIRT1 in autophagy and liver I/R injury is unclear or unknown. This chapter focuses on the background information for liver I/R injury, mitochondrial dysfunction, autophagy, and sirtuins.

The Liver

In humans, the liver is the primary metabolic organ for coagulation factor synthesis, blood filtration, vitamin and mineral storage, production and secretion of bile, and toxic compound detoxification (1). The gross anatomy of the liver can be divided into 4 different lobes (caudate, quadrate, right and left lobes) or eight segments based on the venous, arterial, and biliary systems (Figure 1-1) (2). The liver is composed of
several different cell types including hepatocytes (parenchymal cells), endothelial cells, Kupffer cells, Ito cells, biliary epithelial cells, smooth muscle cells, PIT cells and nerve cells that function in unison to maintain liver function (Table 1-1). Liver lobules are the smallest functional units of the liver (1). Structurally, liver lobules are irregular shaped polygonal prisms that contain portal areas at the corners of adjacent lobules and a central vein (Figure 1-2). Portal areas are regions of connective tissue that contain the portal triad branches, which include the bile duct, portal vein and hepatic artery. Along the central axis of the liver lobule is the central vein for outgoing blood flow. Blood flows through the liver and is processed by hepatocytes that are constructed into cords within each lobule (Figure 1-2). Hepatocytes are multifaceted polarized epithelial cells with distinct bile canalicular or sinusoidal membranes (3). Lateral surfaces engage in cell-cell contact, interact with basal lamina and face the endothelial cells, while the canalicular domains form at lateral cell-cell contacts (1). This unique structure allows for bile and protein trafficking between the blood and hepatic ducts leading to the gallbladder.

Hepatic blood flow is approximately 1,200 ml/min in women and 1,800 ml/min in men, depending on physiological conditions (1). The liver has an extremely high proportion of blood volume that amounts to 10-15% of the total blood content. Blood that passes through the intestines and spleen is delivered to the liver via the hepatic portal vein and accounts for approximately 70% of total blood inflow. Portal blood carries nutrients and contaminants for the liver to filter, process and secrete back into the blood stream (1). The hepatic artery supplies approximately 30% of the total blood inflow, and is highly oxygenated compared to the portal blood supply (1). The portal and arterial blood mix within hepatic sinusoids found within the liver lobule and exits via
central vein that connects to hepatic vein and the inferior vena cava. As the blood passes through the lobule, $P_{O_2}$ within the blood decreases due to hepatocyte metabolism, thus creating zonal differences in $P_{O_2}$ and nutrients within the lobule (Figure 1-2) (4). These zonal differences alter hepatocyte metabolism (Table 1-2). Functionally, zone 1 hepatocytes are located in the periportal region and are specialized for gluconeogenesis, $\beta$-oxidation of fatty acids and cholesterol synthesis, while zone 3 are located in the perivenous region and performs glycolysis, lipogenesis and drug detoxification (5;6). Zone 1 contains the highest blood $P_{O_2}$ and is the least sensitive to ischemic stress, while zone 3 contains the least $P_{O_2}$ and most vulnerable to ischemia (5;7;8).

Liver Surgery And I/R Injury

The American Liver Foundation estimates that liver disease affects over 30 million Americans with 30,000 new cases every year. Liver resection, or hepatectomy, is described as the operative removal of the liver, which can be partial to remove a segment or complete. Liver resection and transplantation surgeries are still the most effective interventions for patients with advanced liver diseases and more patients continue to need these surgical treatments (9;10). A partial hepatectomy can remove up to 70% of the total liver by volume without leading to liver insufficiency post resection (11). Increasing the reduction mass to 90% leads to portal hypertension, microcirculatory ischemia and hepatocellular dysfunction (11). A complete hepatectomy is performed during liver transplantation surgery prior to implantation of the donor liver. Approximately 6,000 liver transplantations and 7,000-10,000 liver resections are
performed annually in the United States making these procedures common treatments for liver disease (9;12-16).

The first liver resection was performed by W. Keen in 1899 to treat hepatic cancer (17). Today, liver resections are performed for living donor transplantation and as a curative treatment for various types of liver diseases including cancer, benign tumors and cystic disease. Liver resection surgery has greatly improved operative mortality rate from <20% to <5% in the last three decades making liver resection a successful and safe way to treat patients with liver disease (18). These improvements are due to a better understanding of liver anatomy, improved surgical techniques, more sophisticated equipment, and advances in perioperative care (19-22). However, damage to the liver parenchymal cells increases the incidence of post resection liver failure and patient mortality (22). Post resection liver failure can occur in as little as 48 hours after surgery and can be identified by deeping jaundice, worsening coagulopathy, encephalopathy, and hyperbilirubinemia, which ultimately leads to mortality (11). Preoperatively, a liver can be assessed for parenchymal function by assessing the Model for End-Stage Liver Disease (MELD) score, which is based on the hepatic synthesis and secretion of bilirubin, creatinine and prothrombin (23-26). Patients with high MELD scores indicate an impaired liver function due to hepatocyte damage, which causes an increase in the incidence of post resection liver failure and mortality to <30% (27-29).

Liver I/R injury can also damage hepatocytes leading to liver dysfunction and patient mortality. Ischemia is defined as the interruption of blood flow carrying oxygen and nutrients to the tissue, while reperfusion is the restoration of the blood flow.
Ischemic temperature can change the causative factors leading to liver dysfunction. During liver transplantation, normothermic explant hepatectomy initially subjects the liver to warm ischemia, which is followed by cold ischemia in a hypothermic preservation medium. Prolonged cold ischemia leads to reperfusion induced endothelial cell dysfunction and Kupffer cell activation, which are the primary factors contributing to liver graft dysfunction (30;31). In contrast, a partial hepatectomy is performed under normothermic conditions and often utilizes vasculature clamping of the portal triad, known as the Pringle maneuver, to minimize blood loss. This creates hepatic inflow occlusion resulting in an inevitable warm I/R event (18). Hepatocyte damage is the major cause of warm I/R injury (32-34). The liver is innately vulnerable to ischemia during inflow occlusion, especially in the zone 3 (perivenous regions) (5;7;8). Prolonging ischemia to one hour can further damage parenchymal cells leading to liver dysfunction (35). Ischemia initially causes hepatocyte damage during inflow occlusion, but reinstitution of blood flow exacerbates hepatocyte damage from the restoration of physiological pH, the accumulation of toxins from intestinal venous congestion during inflow occlusion, and activation of Kupffer cells (5;7;8). Warm I/R injury diminishes adequate postoperative liver remnant function and can be a major cause of morbidity and mortality in patients after liver resection surgery (9;36;37). The mechanisms underlying warm I/R injury to the hepatocytes are multifactorial, including Ca^{2+} deregulation, mitochondrial dysfunction, generation of reactive oxygen and nitrogen species, loss of cellular antioxidants, and stimulation of catabolic enzymes (7;38;39). Warm I/R injury is a primary factor leading to hepatocyte death, liver failure and patient mortality after liver resection surgery and the main focus of this study.
Mechanisms Of Warm I/R Injury

Vasculature clamping causes hepatic inflow occlusion leading to oxygen and nutrient depletion in the liver. The lack of oxygen impairs mitochondrial oxidative phosphorylation and ATP generation. At this point, the glycogen storage is utilized to support anaerobic glycolysis for ATP generation leading to an accumulation of lactic acid that in turn decreases the intracellular pH (5), ultimately leading to tissue acidosis. Tissue acidosis occurs by the accumulation of lactic acid, a buildup of hydrogen ions from the hydrolysis of ATP, and the disruption of hydrogen ions from acidic organelles (5;40;41). Intracellular acidosis conveys cytoprotection against I/R injury in a cyclophilin D mediated manner (42). Cyclophilin D is a peptidyl prolyl isomerase that becomes activated by the reinstitution of physiological pH, which in turn leads to mitochondrial rupture and cell death (42;43). This hepatic reinstatement to physiological pH worsening hepatocyte damage and causing I/R injury is known as the pH paradox (5).

Warm I/R injury can be separated into different phases based on the mechanism for cell death. Necrotic cell death begins after 30 minutes of ischemia at pH 7.4 from ATP depletion, reactive oxygen species (ROS) production at the ischemia/normoxia border, and protease activation (5). Reperfusion induced hepatocyte death increases at three different phases: early, intermediate and late. Early reperfusion injury damages hepatocytes from the first minute and up to 6 hours (44). Necrotic cell death occurs within the first hour of reperfusion from mitochondrial dysfunction and ROS production (7;38;39;45). ROS activate Kupffer cells to promote even higher ROS production that further damages hepatocytes and increases cytokine production (44). Next, the intermediate phase of hepatocyte damage is Kupffer cell-mediated, which occurs after 6 hours of reperfusion. Kupffer cells release ROS, cytokines and chemokines to initiate
neutrophil migration that further damages the liver (5). Lastly, the late phase is mediated by neutrophils and occurs up to 24 hours after ischemia. Neutrophils migrate and become activated to release ROS, cathepsins and elastases to damage and impair the liver (5). Liver damage due to alterations in the metabolism of hepatocytes during the early phase of reperfusion is a factor leading to the intermediate and late phases of warm I/R injury (32-34;46) and the primary focus of this study.

**Mitochondrial dysfunction**

Mitochondrial dysfunction is the causative mechanism contributing to hepatocyte death during early reperfusion injury. Mitochondrial membranes are impermeable to solutes, therefore transport into the mitochondria requires specific carrier proteins. During reperfusion, the mitochondrial impermeability is lost due to the unregulated opening of mitochondrial permeability transition (MPT) pores. Unregulated opening of the MPT pores lead to an influx of solutes up to 1,500 Da into the mitochondrial matrix. This causes mitochondrial swelling, depolarization, uncoupled oxidative phosphorylation, ATP depletion, mitochondrial membrane rupture, the release of pro-apoptotic proteins, such as cytochrome c, that are normally sequestered in the inner membrane, and cell death (7;39;45;47) (Figure 1-3). The MPT pores are located at contact sites between the mitochondrial inner and outer membranes providing a mechanism for rapid changes with the mitochondria. The transient opening of the MPT pores under normal physiological conditions is suggested to regulate Ca^{2+} and ROS within the mitochondria for altering cellular metabolism (48;49). However, the unregulated opening of the MPT pores is a lethal event within <1 hour after reperfusion and termed as the MPT onset (7;38;39;47;50-53).
The protein composition of the MPT pore is still controversial, but is likely composed of several different proteins including adenine nucleotide translocator (ANT) located on the inner mitochondrial membrane, voltage dependent anion channel (VDAC) located on the outer mitochondrial membrane, and cyclophilin D located in the mitochondrial matrix (7) (Figure 1-3). Several other proteins have also been suggested to be involved in the MPT pore including hexokinase and Bcl-2 family members (7). The proapoptotic protein Bax can translocate to the mitochondrial outer membrane to promote the opening of the MPT pore (5). Structural controversy was further enhanced in the finding that VDAC or ANT KO cells can still undergo the MPT onset (54;55). Further studies are required to provide clarity into the structure and formation of MPT pore. Low pH and cyclosporine A, an immunosuppressive agent, suppress the MPT onset by inhibiting cyclophilin D (5). Cyclophilin D causes the conformational change of ANT from the trans to cis form leading the MPT onset during liver I/R injury (42;43;49;56), thus suggesting that ANT is part the MPT pore leading to cell death during liver I/R (Figure 1-3).

Mitochondrial dysfunction and ATP depletion is the causative mechanism leading to necrosis during I/R injury (37;57;58). Ruptured mitochondria release cytochrome c that binds to apoptosis-inducing factor-1 and pro-caspase 9 to form a protein complex, known as the apoptosome, that is ATP dependent to signal for apoptosis through caspase 3 (5;7;59;60). Generation of ATP through glycolysis can play a factor in apoptotic signaling, however the mitochondrial ATPase activity is reversed leading to the depletion of ATP during reperfusion (5). ATP depletion leads to necrosis, while the presence of low ATP induces apoptosis (7;38). Necrotic cell death occurs in <2 hours
after reperfusion suggesting that these hepatocytes are the most sensitive for ATP depletion and mitochondrial dysfunction, which in turn leads to necrosis, Kupffer cell activation, and recruitment of neutrophils for later phases of reperfusion injury (5;7;38).

**Mitochondrial Ca$^{2+}$ and the MPT onset**

Intracellular Ca$^{2+}$ plays a major role in mitochondrial dysfunction, the MPT onset and cell death during liver I/R injury (5) (Figure 1-4). Intracellular compartments containing Ca$^{2+}$ include the cytosol, endoplasmic reticulum and the mitochondria. The depletion of ATP during ischemia impair and alter ion pumps that balance intracellular concentrations of Ca$^{2+}$, Na$^+$, and H$^+$. During ischemia, the accumulation of H$^+$ leads to the activation of the Na$^+$/H$^+$ exchanger in the plasma membrane, while Na$^+$/K$^+$ ATPase is impaired due to ATP depletion leading to an accumulation of Na$^+$ into the cytosol (61;62). Na$^+$, in turn, activates the Na$^+$/Ca$^{2+}$ exchanger in the plasma membrane to accumulate Ca$^{2+}$ in the cytosol (62-64). This process is accelerated upon reperfusion due to the removal of H$^+$ ions by the Na$^+$/H$^+$ and Na$^+$/Ca$^{2+}$ exchangers (61-65). Furthermore, endoplasmic reticulum Ca$^{2+}$ storages are also affected during I/R by the inhibition of Ca$^{2+}$ reuptake, and the enhancement of Ca$^{2+}$ release through ryanodine receptor (61;62;65). These alterations in ion balance lead to an accumulation of cytosolic Ca$^{2+}$ concentrations, but cytosolic Ca$^{2+}$ does not lead to hepatocyte I/R injury (5;39;45).

Mitochondrial Ca$^{2+}$ overload is the key factor that initiates the opening of the MPT pore, which is followed by generation of ROS (39;45). The increased cytosolic Ca$^{2+}$ is taken up into the mitochondria via the mitochondrial Ca$^{2+}$ uniporter, which requires polarized mitochondria to facilitate inflow. Mitochondria become repolarized within 5 minutes of reperfusion, which drive the Ca$^{2+}$ influx. The excessive influx of Ca$^{2+}$ into the
mitochondria activates cyclophilin D leading to the MPT onset (42;43). Mitochondrial 
Ca^{2+} chelation suppresses hepatocyte necrosis and ROS production suggesting the 
mitochondrial Ca^{2+} overload occurs prior to ROS generation (39;45). Furthermore, pH 
dependent reperfusion induced death occurs in the absence of oxygen suggesting that 
Ca^{2+} overload is independent of oxygen and mitochondrial ROS production leading to 
the MPT onset and cell death (5).

**Proteases, lysosomes and I/R injury**

Calpains are a family of non-lysosomal cysteine proteases that degrade 
intracellular proteins in the cytosol, cytoskeleton, ER and mitochondria (50;53;66). Calpains are expressed as two isoezymes that become activated through different 
concentrations of Ca^{2+} as indicated by their nomenclature: 3-50 μmol/L (μ-calpain) and 
400-800 μmol/L (m-calpain). Elevation in Ca^{2+} concentrations during I/R leads to the 
activation of calpains (5;50;53) and the degradation of the endogenous calpain inhibitor 
calpastatin (62). Inhibition of calpains through pharmacological or genetic approaches 
protect against I/R injury to the brain, heart, liver, kidney and intestines (62;67-69). 
Calpain activation can lead to the degradation of lysosomal membrane proteins leading 
to lysosomal membrane destabilization (70-72). Calpain localizes to the lysosomal 
membrane after the onset of ischemia, and subsequent spillage of cathepsins into the 
cytoplasm occurs (70;72), however the mechanism is poorly understood.

Lysosomes, nicknamed “suicide bags” by Christian Rene de Duve, are 
cytoplasmic membrane bound vacuoles that degrade macromolecules. Lysosomes 
contain over 50 soluble hydrolytic enzymes including proteases, lipases, nucleases, 
glycosidase, phospholipases, phosphatases and sulfatases that have a maximal activity
at low pH within the vacuole that degrade proteins, lipids and organelles (71). Cathepsins are a family of proteases with over 11 members that localized to the lumen of the lysosome (73) but recent reports have observed cathepsins in the cytosol during hepatic stressors such as I/R and acetaminophen toxicity (74;75). Cathepsins are divided into three groups based on structure and catalytic type: serine (cathepsins A and G), aspartyl (cathepsins D and E) and cysteine (cathepsin B, C, F, H, K, L, O, S, W and Z) (73). Cathepsin activity is optimal under acidic conditions (pH 5-5.5) within the lysosome, however cathepsins B and D are stable at physiological pH with cathepsins D proteases activity at 42% (76;77). Lysosomal leakage and rupture has been proposed to release hydrolytic enzymes into the cytoplasm after oxidative stress and I/R (78-80).

During I/R, pharmacological inhibition of cathepsins reduces cerebral I/R injury (81-83). During liver ischemia, hepatic lysosomal rupture is an event that proceeds the MPT onset (52), which may be mediated by calpain activation and mitochondrial dysfunction. The mechanism for lysosomal destabilization during I/R remains unclear and controversial with proposed mechanisms being (A) ROS induced lipid peroxidation (71), (B) increased cytosolic Ca^{2+} concentrations that activate phospholipase A2, sphingomyelinase and phospholipase C to generate lipid metabolites (84) and (C) calpain mediated cleavage of lysosomal associated membrane proteins that stabilize the membrane (72). Several studies have shown that cathepsins translocate into the cytosol during I/R (71;73;76;83), lending calpain mediated lysosomal membrane permeability an area of research that requires more investigation.

**Surgical And Pharmacological Strategies To Suppress Liver I/R Injury**

During liver resection, inflow occlusion is often performed to minimized blood loss. Different methods of inflow occlusion include continuous or intermittent Pringle
maneuvers, segmental clamping, and total hepatic vascular exclusion. Each method is uniquely different, however the common factor is vasculature clamping that exposes the liver to an I/R event. In addition, liver transplantation surgery requires explant hepatectomy prior to implant of the donor liver, which subjects the liver to warm ischemia that can damage hepatocytes, hence the importance to develop protective strategies against liver I/R injury (85).

Hypothermia has been used for decades to decrease I/R injury during liver transplantation, however the role of hypothermia in I/R injury during partial hepatectomy has not been well established (9;85). The only strategy to reduce hepatic I/R injury is ischemic preconditioning (IPC) and intermittent clamping, however it is only beneficial for limited duration of ischemia (9;37;46;85;86). IPC is the short and repeated exposure to ischemia followed by reperfusion. The first human trial of IPC was performed by Clavien and showed beneficial effects using 10 minute periods of ischemia followed by 10 minutes of reperfusion by a decrease in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) but no significant change in the morbidity and mortality rates (87;88). IPC is a promising approach in several different organs and animal models preventing I/R injury (18;85), but some studies contradict these findings (89). IPC provides protection in several different ways including increasing ATP levels, regulating pH, balancing Na\(^+\) and Ca\(^{2+}\), suppressing Kupffer cell activation, enhancing autophagy, and promoting synthesis of multiple stress response proteins (5;90;91). The disadvantages of IPC are repetitive clamping induced vasculature damage and stress to the target organ (92). Furthermore, IPC is not recommended for older patients.
undergoing liver resection, and more importantly the IPC provided benefits only minimally protect against warm hepatic I/R injury (93).

Therapeutics strategies to suppress liver I/R injury have included antioxidant therapy, steroids, Ca\(^{2+}\) channel blockers, immunosuppressants, and protease inhibitors but demonstrated no evidence of clinical benefit (9;94). Antioxidant therapies include N-acetylcysteine, post ischemic glutathione administration, Sevoflurane, S-adenosylmethionine, Vitamin E and Trimetazadine. Administration of these drugs is targeted toward reperfusion induced ROS production by increasing antioxidant enzymes and metabolites including Superoxide dismutase and glutathione levels (94). Studies using these agents have either not made it to clinical trials or failed to provide protection against liver I/R injury (94). Prednisolone, a glucocorticoid steroid, and immunosuppressants target the cytokine response during the intermediate and late phases of I/R injury (94;95). Ca\(^{2+}\) channel blockers (CCB) is another strategy to reduced liver I/R injury by targeting the Ca\(^{2+}\) imbalance that occurs during I/R. CCB has been studied in experimental models but clinical data is absent (94;96;97). Protease inhibition is focused on suppressing the activation of catalytic enzymes that are activated during I/R such as cathepsins and calpains (50;51;67-69). Altogether, these current therapeutic strategies to prevent liver I/R injury remain ineffective. The multifactorial nature and complexity of liver I/R injury creates a significant therapeutic challenge for targeting a specific mechanism to convey protection, thus new strategies are required.

**Autophagy**

Autophagy is a conserved lysosome dependent catabolic process that eliminates protein aggregates and damaged organelles (98). Autophagy was first described in 1963 by Christian de Duve and is defined as a cell “self-eating” mechanism (99). There
are 3 different types of autophagy: chaperone mediated autophagy, microautophagy and macroautophagy (Figure 1-5). Chaperone mediated autophagy is the selection of soluble cytosolic proteins that are targeted to the lysosome and directly translocate across the lysosomal membrane for degradation (100). Microautophagy involves the direct engulfment of cytoplasmic cargo by the lysosome (101). Macroautophagy is the focus of this study and referred to as Autophagy hereafter.

Autophagy is a sequential process that begins with the initiation and formation of an autophagosome, a double membrane vacuole, which sequesters cargo for transported to the lysosome for fusion and degradation (Figure 1-5). Canonical autophagy relies on the recruitment of multiple autophagy related protein (ATG) complexes onto a cup shaped membrane assembly site termed the phagophore. Non-canonical autophagy is poorly understood but involves the formation of autophagosome independent of some ATG proteins (102). Autophagy can be a selective or non-selective process for sequestering cargo. Selective autophagy can include the removal of specific proteins and organelles: peroxisomes (pexophagy) (103), mitochondria (mitophagy) (104), ribosomes (ribophagy) (105), endoplasmic reticulum (reticulophagy) (106), and nucleus derived material (nucleophagy) (107), while there is no current evidence for the golgi apparatus (golgi-phagy) (108). Autophagy maintains cellular homeostasis and normal physiology the orchestration of several proteins at the different stages of autophagy: initiation, autophagosomal maturation, and lysosomal fusion.

**Autophagy Initiation**

Autophagy occurs at a basal rate in most cells to maintain homeostasis (109), or can be stimulated in pathological and physiological states including starvation (98). Autophagy is tightly regulated to establish homeostasis during stress for survival, while
impaired regulation and hyperactivation can lead to cell death (47;110). The most studied autophagy initiation mechanisms are the mammalian target of rapamycin (mTOR), and adenosine monophosphate-activated protein kinase (AMPK) pathways, which are stimulated by nutrient availability and cyclic adenosine monophosphate (cAMP) levels, respectively (98) (Figure 1-6). These pathways can initiate autophagy by up regulating transcript of autophagic genes and the recruitment of the autophagic machinery to the phagophore site for autophagosome generation (98;111-113).

Nutrient and amino acid deprivation promote autophagy in a variety of cell types, while fasting and caloric restriction promotes in vivo autophagy (114). Nutrient rich conditions promote cell growth and proliferation through the phosphoinositide 3-kinase (PI3K) class I/Akt pathway, which in turn actives mTOR, a serine/threonine protein kinase, leading to autophagy inhibition (112). Deprivation of growth factors, insulin or amino acids converges at mTOR to initiate autophagy (114). The upstream signals leading to mTOR inhibition and autophagy initiation is dependent on method of deprivation (112). Two different mTOR complexes (mTORC) are found in mammalian cells: mTORC 1 and mTORC2 (114-116). Akt phosphorylation regulates mTORC1 and mTORC2 through raptor and rictor, respectively (112;114;116). Inhibition of either mTORC can lead to enhanced autophagy. Inhibition of mTORC2 is involved in FOXO3A transcription of autophagy genes, while mTORC1 inhibition leads to a dephosphorylation of transcription factor EB (TFEB), UNC-51 like kinase (ULK1/2) and ATG13. mTORC1 inhibition facilitates (A) nuclear localization of TFEB, a master regulator for lysosomal biogenesis and autophagy gene transcription (111) and (B) ULK1/2 autophosphorylation, which in turn phosphorylates ATG13 and FIP200 (117).
The ULK-ATG13-FIP200 complex localizes on phagophores during the early nucleation process of the autophagosome (118). The function and localization of ULK-ATG13-FIP200 is suggested to be involved in the recruitment of autophagic machinery for autophagosome generation, and to activate Beclin1-PI3K class III (PI3KIII) complexes for phosphatidylinositol-3-phosphate (PI3P) insertion into the autophagosomal membrane (112;119).

AMPK signals autophagy initiation in the presence of low ATP and high cAMP levels during nutrient deprivation and hypoxia (120). AMPK can initiate autophagy through mTOR inhibition dependent and independent mechanisms. mTOR inhibition dependent mechanisms include AMPK mediated phosphorylation of A) the raptor subunit at Ser\textsuperscript{722} and Ser\textsuperscript{792} leading to mTORC1 binding to 14-3-3, which in turn suppresses kinase activity, or B) Tuberous Sclerosis Complex 2 (TSC2) at Ser\textsuperscript{1387} that inhibits Rheb, an upstream mTOR activator (121). mTOR independent autophagy initiation occurs by AMPK mediated phosphorylation of ULK1 and Beclin1 to promote autophagy (122;123).

The Beclin-PI3KIII complex is another mechanism that can regulate autophagy. The PI3KIII complex contains the vacuolar protein sorting 34 (VPS34) protein, and p150 that anchors the complex to membranes via a myristoylation on the N terminus (124). VPS34 is a class III lipid kinase that catalyzes phosphatidylinositol (PI) to PI3P for vesicle trafficking (125). PI3P insertion in the autophagosomal membrane leads to membrane curvature and recruitment of proteins containing a PI3P binding domain (126). VPS34 has a variety of cellular functions including retrograde trafficking from endosomes to the Golgi, and phagosome maturation (125). Beclin 1, the mammalian
homolog of ATG6, can form a complex with VPS34 and recruit additional autophagy proteins such as Rubicon, Ambra-1, ATG14L, and UVRAG that lead to the regulation of PI3P synthesis (123). Furthermore, Bcl-2 can compete against VPS34 for the Beclin 1 binding sites to inhibit autophagy (127). ATG14L, named barkor, recruits the Beclin-PI3KIII complex to the ER for autophagy initiation (126). UVRAG is another protein that stimulates the complex activation, but competes with ATG14L for the coil-coil domain of Beclin 1 (124;128). UVRAG-Beclin-PI3KIII complexes function in both autophagosomal formation and maturation (124). Ambra1 is activated by ULK phosphorylation and binds to the Beclin-PI3KIII complex for dynein motor complexes assembly for translocation to the ER during autophagy initiation (129). Rubicon inhibits the production of PI3P by binding to UVRAG and VPS34 to prevent PI3P synthesis (124;130).

**Autophagosomal Membrane Elongation And Maturation**

Autophagosomes are double-bilayer membrane vacuoles with a diameter of 300-900 nm that sequester cellular components (98). The production of autophagosomes is through several different ATG proteins and the Beclin-PI3KIII complex (98). After autophagy initiation, autophagosomal elongation proteins (ATG4B, ATG7, ATG3, ATG10, ATG12, ATG5, and ATG16) are recruited to a phagophore assembly site (131). Autophagosomal generation can occur at several sites including the ER, mitochondrial outer membrane, and Golgi apparatus (98). Microtubule associated protein 1 light chain 3 (LC3) has an essential role in autophagy for membrane elongation, recruitment of adaptor proteins, and fusion (109). Among the 4 different isoforms, LC3A, LC3B, GATE-16 and GABARAP, LC3B is the most widely used for studying autophagy (132). The autophagy machinery is recruited to the phagophore assembly site to process the cytosolic LC3 (pro-LC3) into a membrane bound LC3 (LC3-II), which is located on the
inner and outer membranes of the autophagosome (Figure 1-7) (109). LC3 is found in 3 forms within a cell: pro-LC3, LC3-I and LC3-II. In cells, LC3-II is bound to the membrane of maturing autophagic vacuoles through two ubiquitin like systems, ATG8 and ATG12 (133). In both systems, ATG7 acts as the E1 like activating enzyme, while the E2 like conjugating enzymes differs: ATG3 for ATG8 and ATG10 for ATG12. LC3 is expressed as a precursor and is cleaved by the cysteine protease ATG4B, which removes the C terminal arginine to expose a glycine residue prior to lipidation (134). The glycine is primed by ATP hydrolysis and ATG7 to form acyl adenylated LC3 known as LC3-I (134). The acyl adenylation is then attacked by a thiol group from an internal cysteine residue of ATG7 to establish a thioester bond between the glycine and cysteine residues (133;134). In the ATG12 system, the thioester bond is transferred to an internal cysteine residue in ATG10 or ATG3 in the ATG8 system (135;136). ATG10 acts as the conjugating enzyme to target ATG5. The final transfer of ATG12 to ATG5 establishes an isopeptide bond between a glycine residue of ATG12 and an internal lysine residue on ATG5 (133). The ATG12-ATG5 conjugate associates with ATG16 through the N-terminal domain of ATG5 and acts as an E3 like ligase for LC3-I and phosphotidylethanolamine (PE) in the membrane to generate the lipidated LC3 known as LC3-II (137).

**Lysosomal Degradation**

Mature autophagosomes fuse with lysosomes for degradation. The hydrolytic nature of the lysosome was previously described. To facilitate membrane tethering autophagosomes often utilize Rab proteins to mediate autophagosomal-lysosomal fusion (138). Rabs are Ras-like GTPases that localize on the membrane of specific cellular compartments and are ideal candidates for the regulation of membrane fusion.
Specifically, Rab7 has been shown to play a role in autophagosome-lysosome fusion through to its ability to interact with the FYCO1, which contains a FYVE domain to bind PI3P to facilitate fusion (139). Furthermore, UVRAG-Beclin-PI3KIII complex can interact with the class C vacuolar protein sorting complex and enhance Rab7 facilitated fusion (140). Several studies are currently focused on the identification of Rab proteins involved in autophagy; while a detailed molecular mechanism remains unclear, thus intensive studies are required for fully understanding the role of Rab proteins in membrane fusion (139). Vesicular membrane tethering is mediated by soluble N-ethylmaleimide-sensitive fusion factors (SNARES), which are transmembrane proteins that can assemble into high-affinity trans complexes between two opposing membranes to drive the fusion process (140;141). Membrane tethering occurs through the formation of a four-helix bundle, which is composed of three Q-SNARES and one R-SNARE. Vesicular associated membrane protein 7 (VAMP), VAMP8 and Vti1 are SNARE proteins that mediated lysosomal fusion with autophagosomes (140).

**Mitochondrial Autophagy (Mitophagy)**

Mitochondrial autophagy, known as mitophagy, is the only known mechanism to remove damaged or dysfunctional mitochondria. Autophagy regulates mitochondrial turnover, which occurs every 15 to 25 days (142). The impairment of mitophagy can result in the accumulation of abnormal or damaged mitochondria, which in turn causes uncontrolled ROS production (143). Mitophagy can be divided into three different types: Type I, Type II and Type III (104). The molecular and biochemical differences between these types require further investigations. Type I occurs at the phagophore assembly site and requires PI3KIII signaling, Type II requires mitochondrial depolarization followed by LC3 accumulation on the mitochondrial surface, and Type III is called
“micromitophagy” which involves the formation of mitochondria-derived vesicles (MDVs) enriched in oxidized mitochondrial proteins that bud off and transit into multivesicular bodies (104). Overall, Type I and Type II mitophagy can engulf an entire mitochondrion for removal, while Type III is a type of quality control mechanism to selectively remove damaged and oxidized mitochondrial components (104).

Several proteins have been identified to induce mitochondrial autophagy. Two of the earliest mammalian molecules identified were PTEN-induced putative kinase protein 1 (PINK1) and PARKIN in neuronal cells in the substantia nigra (144;145). PINK1 translocates to the mitochondria and is cleaved by the mitochondrial serine protease presenilin-associated rhomboid like protein (PARL) in polarized mitochondria (146). The PARL activity and the cleavage of PINK1 are impaired in depolarized mitochondria leading to full length PINK1 translocation to the outer mitochondrial membrane. Full length PINK1 can bind to Beclin 1 and recruits Parkin, an E3 ubiquitin ligase, for outer mitochondrial membrane protein ubiquitination that facilitates selective mitochondrial autophagy (147;148). Transcription factor p62 can act as a linker protein to bind autophagic cargo and autophagosomes through ubiquitin and LC3 binding domains, respectively (149). Recently, p62 has been shown to recognize dysfunctional mitochondria through Parkin induced VDAC ubiquitination on the outer mitochondrial membrane, however the role of p62 in mitochondrial autophagy is unclear (150;151). Mitochondrial receptors BNIP3/NIX or FUNDC1 may also be involved in mitochondrial autophagy (152). Bcl2/adenovirus EB 19-kDA interacting protein 3 (BNIP3) and FUNDC1 induces autophagy by binding to LC3 through a WXXL motif to remove
mitochondria (152-154). Several more investigations are required to fully clarify the protein mechanisms and types of mitophagy within mammalian cells.

**Autophagy Suppresses Liver I/R Injury**

A novel approach to circumvent liver I/R injury is to enhance autophagy, which in turn removes the damaged mitochondria that cause hepatocyte death and liver dysfunction (47). Liver I/R leads to the accumulation of dysfunctional mitochondria undergoing the MPT onset, which cannot be removed due to an impaired autophagic response (51-53). Autophagy allows for the removal of these damaged mitochondria, the replenishment of amino acids into the cytosol and the suppression of mitochondrial ROS generation. The first study investigating autophagy and I/R injury occurred in 1981 using rat livers subjected to sixty minutes of ischemia (155). The description of an impaired autophagic response was observed and described as “livers contain autophagolysosomes with discontinuous membranes and a decreased intravascular electron density” (155). In 1983, rats treated with leupeptin and pepstatin (lysosome protease inhibitors) diminished the hepatic damaged caused by 20 minute of ischemia providing evidence that lysosomal autophagy may have a role in I/R injury (156). In 1992, the first I/R induced hepatic autophagic response was proposed in a hepatocytes was describes as vesicular bodies with unchanged cytoplasmic regions and organelles (157), an autophagosome. At this point, the detailed biochemical mechanisms causing autophagy were still limited with primary studies using imagining techniques. A slew of autophagy papers detailing biochemical measures and molecules to regulated autophagy were published between 1998-2004 (106;133;135;137;158). In 2008, autophagy was shown to suppress I/R injury leading to the first postulation that targeting autophagy could be used as a cytoprotective mechanism against warm liver I/R injury
Since 2008, autophagy stimulation has been shown to be a cytoprotective mechanism to circumvent warm I/R injury through several different approaches: pharmacological (cisplatin (159), carbamazepine (50), and lithium (160)), surgical (IPC (161-163)), and nutritional (Fasting (164)).

Sirtuins

Histone deacetylases (HDACs) are a class of enzymes that modify proteins by removing an acetyl group (O=C-CH3) from an ε-N-lysine residue. HDACs are subdivided into classes based on their sequence homology to the original yeast enzymes and domain organization: Class I, Class II, Class III, and Class IV. Silent mating type information regulation 2 (SIR2) genes are the yeast homolog for the mammalian sirtuin proteins. Sirtuins are class III HDACs dependent on NAD⁺ for enzymatic activity (165;166). Sirtuins are involved in regulating several cellular processes including transcription, mitochondrial biogenesis, oxidative phosphorylation, and autophagy (167;168). There are seven different isoforms of sirtuins (SIRT1-7) that localize to different subcellular compartments, but each contain a conserved NAD⁺ deacetylase domain; however not all sirtuins perform protein deacetylation (Table 1-3). The highly divergent amino and carboxyl terminal sequences are involved in substrate recognition of each sirtuin isoform, and play a role in enzyme activity (168). Sirtuins are suggested to be metabolic sensory enzymes that can regulate metabolism with their activation based on the NAD⁺ levels (167-170). Currently, sirtuins are being studied for their therapeutic potential in several different diseases including cancer, neurodegeneration, cardiovascular disease, diabetes and obesity.
Sirtuin Enzymatic Activity

In 1984, SIR2 genes were first identified as genes involved in the regulation of mating types in budding yeast, *Saccharomyces cerevisiae* (171). Over two decades passed until the enzymatic activity of SIR2 proteins was demonstrated to catalyze a pyridine nucleotide transfer reaction in both bacteria and mammals by a report showing the transfer of $^{32}$P from $^{32}$P-NAD to bovine serum albumin (165). This proposed the first important role of *SIR2* as an ADP-ribosyltransferase (165;172). Next, SIR2 deacetylase activity was identified as evolutionarily conserved and uniquely dependent on NAD$^+$ to establish chromatin silencing structures *in vivo* by histone 4 deacetylation (173). Early studies demonstrated that extra copies of *SIR2* genes increased lifespan in simple organisms (174-176), while decreasing *SIR2* blocked the caloric restriction induced lifespan extension (176). Today, mammalian sirtuins are known to play a key role in regulating several mechanisms including autophagy, mitochondrial biogenesis, and β-oxidation during caloric restriction and aging (177;178).

Sirtuins are unique among other HDACs enzymes due to their insensitivity to the potent HDAC inhibitor, Trichostatin A, and requirement for NAD$^+$ as a cosubstrate (168;179;180). The sirtuin conserved catalytic domain can contain up to 270 amino acid residues and form a reverse Rossmann fold, and zinc ribbon (181;182). The Rossmann fold binds to the phosphate and two ribose groups of NAD$^+$, while the zinc ribbon utilizes a zinc atom to stabilize two cysteine residues within the conserved catalytic domain of the sirtuin (168). To catalyze deacetylation, sirtuins require an acetylated lysine substrate to bind within a cleft adjacent to the Rossmann fold, thus forming a ternary complex to facilitate a conformational change that internalize the acetyl-lysine residue.
and promotes NAD$^+$ binding (168) (Figure 1-8). Sirtuins cleave a glycosidic bond to separate nicotinamide and the ribose moieties of NAD$^+$ to form nicotinamide and an enzyme-ADP-ribose intermediate (183). Sirtuins transfer the acetyl group to an ADP ribose to generate 2’-O-acetyl-ADP-ribose and a deacetylated substrate (184). Finally, nicotinamide, 2’-O-acetyl-ADP-ribose, and the deacetylated substrate are released.

**Sirtuin Regulated Liver Functions**

**Sirtuin 1**

SIRT1, the most studied sirtuin, is localized to the cytosol and the nucleus for deacetylation of histone and non-histone substrates (185) (Table 1-3). SIRT1 is a pleotropic protein involved in regulating circadian rhythms (186-188), autophagy (164;189-192), gluconeogenesis (193;194), fatty acid oxidation (193;195), mitochondrial biogenesis (193;196;197), cell proliferation (198;199) and antioxidant defense (190). SIRT1 null and catalytic domain depleted transgenic mice were embryonic and postnatal lethal demonstrating the essential nature of SIRT1 activity (200). SIRT1 studies are rapidly accelerating as potential therapeutics in muscle, liver, brain, kidney, heart, and lung diseases, as well as aging, diabetes and cancer (194;195). SIRT1 substrate deacetylation and metabolic regulation is tissue specific, we are focusing on the SIRT1 mechanisms that regulate liver functions.

In the liver, SIRT1 regulates gene transcription for gluconeogenesis and fatty acid oxidation through deacetylation of multiple transcription factors: peroxisome proliferator-activated receptor-$\gamma$ coactivator 1$\alpha$ (PGC-1$\alpha$) (194), CREB regulated transcription coactivator 2 (CRTC2) (201), Forkhead transcription factors (FOXO) (202), fibroblast growth factor (FGF21) (203) and signal transducer and activator of transcription (STAT3) (204). Cholesterol levels are positively regulated by SIRT1 by the
liver X receptor α (LXRα), a nuclear receptor, for lipid homeostasis (205). Deacetylation of LXRα activates gene transcription of ABCA1 that mediates high-density lipoprotein (HDL) synthesis and HDL reverse cholesterol transport (206). Deacetylation of sterol regulatory element-binding protein (SREBP-1) leads to an increased lipid metabolism in mice exposed to ethanol (207). SIRT1 regulates several different cellular metabolic pathways, hence SIRT1 investigations for therapeutic potential in several liver diseases including alcoholic and nonalcoholic induced fatty liver diseases, hepatocellular carcinoma, cirrhosis, diabetes, and I/R injury.

**Sirtuin 3 and Sirtuin 5**

SIRT3 is an NAD⁺ dependent deacetylase that is localized to the mitochondria and nucleus (208;209). SIRT3 is the key regulator for energy homeostasis through mitochondrial protein deacetylation that regulates ATP production, and β-oxidation (208;210). Circadian oscillations in mitochondrial NAD⁺ regulate SIRT3 activity to modulate the mitochondrial oxidative enzymes and respiration (211). SIRT3 plays a key role in mitochondrial ATP production by deacetylation of the NDUFA9 subunit of Complex 1 (212). Furthermore, SIRT3 mediates hepatic mitochondrial ketone production by 3-hydroxy-3-methylglutaryl-CoA 2 deacetylation, which is the rate limiting step in β-hydroxybutyrate synthesis, a ketone (213;214). Pathological and physiological conditions can alter mitochondrial metabolism through SIRT3. Under basal conditions SIRT3 null mice do not appear phenotypically different, however fasting leads to an accumulation of triglycerides and fatty-acid intermediates (215). Under these conditions, long chain acetyl coenzyme A dehydrogenase (LCAD) was identified as a substrate of SIRT3 deacetylation (215). Detoxification of acetaminophen metabolites occurs in the
mitochondria through mitochondrial aldehyde dehydrogenase 2, which is a direct substrate of SIRT3 (216). SIRT3 studies are rapidly accelerating and an exciting new area of research for potential therapeutics.

SIRT5 is an NAD\(^+\) dependent deacetylase that functions for protein deacetylation (217), desuccinylation (218), demalonylation (218), and deglutarylation (219). SIRT5 is localized in the mitochondrial matrix (217) to regulate carbamoyl phosphate synthase 1 (CPS1) activity (217-219). CPS1 is the rate limiting enzyme for the urea cycle and ammonia clearance. Recently, a second function of SIRT5 has been proposed in the livers, Urate oxidase (UOX) can bind to SIRT5 for deacetylation and activation to catalyze urate to allantoin during purine catabolism (220). Several more studies are required to elaborate the role of SIRT5 in the liver mitochondrion.

Other sirtuins

SIRT2, SIRT4, SIRT6 and SIRT7 are the least studied sirtuins involving liver diseases. SIRT2 is reported to be involved in tumor development for hepatocellular carcinoma (HCC). Up regulation of SIRT2 deacetylates glycogen synthase kinase-3\(\beta\) in HCC cell lines to advances tumor development, motility and invasiveness (221). SIRT4 is up regulated in the liver of rats fed a high fat diet and suggested to localize to the cytoplasm to lead to development of insulin resistance and fatty liver disease (222). Interestingly, SIRT4 KO increases fatty acid oxidation through up regulating the function of SIRT1 suggesting SIRT4 is a negative endogenous regulator of SIRT1/PGC1-\(\alpha\) (223). SIRT6 can down regulate glycolysis by interacting with the transcription factor hypoxia-inducible factor 1\(\alpha\) (HIF1\(\alpha\)) to suppress genes transcription (224). SIRT6 deficient mice develop hepatic steatosis and an increase in SIRT6 prevents liver
dysfunction caused by hepatic steatosis (225). SIRT7 regulates lipid metabolism through toll like receptor 4 gene expression for fatty acid uptake (226). Overexpression of SIRT7 promoters synthesis of ribosomal proteins by silencing gene expression and found to be involved in ER stress and fatty liver disease, however SIRT7 mechanistic data is unknown (227).

**Endogenous And Exogenous Sirtuin 1 Regulators**

As SIRT1 is the most studied sirtuin, the regulation mechanisms described here will focus on the investigations identifying the regulation of SIRT1 activity. SIRT1 binding affinity can be regulated by endogenous and exogenous factors (Figure 1-9). Protein-protein interactions, and post translational modifications are endogenous mechanisms that regulate SIRT1 activity. Active regulator of SIRT1 (AROS) is the only known SIRT1 activating protein to directly bind to SIRT1 between amino acids 114-217 for activation (228;229), however the AROS-SIRT1 enhanced deacetylation is controversial and may due to *ex vivo* conditions (230). Deleted in Breast Cancer-1 (DBC1) inhibits SIRT1 activity by binding to the catalytic domain (231). Nutrient starvation leads to a decrease in DBC1 expression and elevated SIRT1 activity, while mice fed a high fat diet lead to elevations in DBC1 and impaired SIRT1 deacetylation leading to non-alcoholic liver steatosis (232). AMPK and PKA phosphorylate SIRT1 at Thr<sup>344</sup> to dissociate the DBC1-SIRT1 complex leading to SIRT1 activation and deacetylation of p53 without an elevation in NAD<sup>+</sup> levels (233-235).

SIRT1 phosphorylation regulates function (236) and several other kinases are involved in phosphorylation of SIRT1 besides AMPK (233) and PKA (233). Phosphorylation on Ser<sup>27,74</sup> and Thr<sup>530</sup> by c-Jun N-terminal kinase 1 (JNK1) increases nuclear localization of SIRT1 and enzymatic activity (237), while JNK2 phosphorylation
on Ser\(^{47}\) leads to protein degradation (238) suggesting that additional mechanisms may play a role in regulating JNK/SIRT1 pathway. Dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK) phosphorylates Thr\(^{522}\) to enhance the release of nicotinamide and the deacetylated substrate to increase enzymatic turnover (239). Protein kinase CK2 phosphorylates Ser\(^{659}\) and Ser\(^{661}\), however no cellular alterations were reported (240;241). Cyclin dependent kinases (Cdk) can phosphorylate Ser\(^{540}\) and Thr\(^{530}\), which is linked to a repressed SIRT1 activity and inhibition of cell proliferation (236). Other conserved phosphorylation sites shown with no mechanistic data are Ser\(^{173}\), Thr\(^{544}\), Thr\(^{719}\) and Thr\(^{747}\) (236).

Sumoylation is a reversible post translational modification in proteins termed small ubiquitin-related modifiers (SUMOs) and are covalently linked to lysine residues. Rat and human SIRT1 contains a sumoylation at Lys\(^{734}\), which is not conserved in mice (242). Sumo-SIRT1 occurs at Lys\(^{379}\) and increases SIRT1 activity and nuclear localization undergoing oxidative stress in mouse cardiac tissue (242;243). Mouse liver studies have not yet characterized a sumoylation site on SIRT1.

Pharmacological activation of SIRT1 can be achieved through small allosteric activators, which have been used in several different models (244-246). The first SIRT1 activator discovered was Resveratrol (RSV), a plant polyphenol, found in red wine (247). The direct effects of RSV with SIRT1 in vitro have been a controversial topic. Studies show that RSV inhibits phosphodiesterases to provide changes in cellular metabolism in addition to enhancing NADH dehydrogenase (Complex I) activity to increase mitochondrial oxidative phosphorylation (248-250). Since the discovery of RSV additional compounds have been synthesized to improve the specificity for SIRT1 with
lower $K_m$ and higher $EC_{50}$ for the SIRT1 substrates including SRT1720 (251). A single amino acid residue Glu$^{230}$ located on the N terminal of SIRT1 is critical for all SIRT1 activating compounds (STACs) (251). Sirtinol is a general sirtuin inhibitor has a human SIRT1 $IC_{50} = 60 \, \mu M$, while human SIRT2 $IC_{50} = 48 \, \mu M$ implicating that Sirtinol induced effects may not be exclusively related to SIRT1 (252). Ex527 is a more specific sirtuin inhibitor with human SIRT1 $IC_{50} = 0.38 \, \mu M$ and human SIRT2 at $IC_{50} = 32.6 \, \mu M$ (253). Furthermore, docking analysis predicts Sirtinol and Ex527 bind to the catalytic domain of SIRT1, but Sirtinol docks to both SIRT1 and SIRT2, while Ex527 binds to SIRT1 alone (253). MicroRNAs have also been suggested to inhibit SIRT1 activity and expression (254;255).

**Activation Of SIRT1 Induces Autophagy**

Early evidence supporting the role of sirtuins in autophagy was the observation that SIR2 gene products tightly regulated caloric restriction induced life extension (176;256). In the following years, SIRT1 was proposed to regulate multiple steps in the autophagic process. SIRT1 induced autophagy was first proposed by Toren Finkel (189) by using SIRT1 null MEF cells demonstrating that SIRT1 was essential for autophagy under both basal and nutrient deprivation conditions to enhance autophagosome generation (189). Next, caloric restricted mice displayed cytoprotection against I/R injury in the heart and kidney, which were mediated by SIRT1 deacetylation of FOXO1 and FOXO3A to increase Rab7 (257-259) and Bnip3 expression (260), respectively. To explore pharmacological avenues, HCT116 and PC3-II cell lines treated with RSV induced autophagy in an SIRT1 dependent manner through AMPK (261;262). Taken together these initial studies, provided ample evidence to support the role of SIRT1 in
autophagy, henceforth numerous cell lines and tissue types have shown SIRT 1 induced autophagy, however the mechanisms remain unclear.

The importance of SIRT1 and autophagy was noted by SIRT1 homozygote knock out mice bearing a resemblance to ATG5 knock out mice (263), which is probably related to the prenatal mortality (200). SIRT1 activity modulates autophagy by two proposed mechanism (A) enhancing gene expression and (B) directly interacting with components of the autophagy machinery. The nuclear/cytosolic localization of SIRT1 can account for either mechanism to stimulate autophagy (185). FOXO family transcription factors are the main focus for the SIRT1 induced autophagy through FOXO1 regulation of Rab7 (190), and FOXO3A up regulation of ULK, Beclin1, VPS34, BNIP3, ATG12, ATG4B, and LC3 (264). Another transcription factor target of SIRT1 is p53, which has dual roles in autophagy regulation based on localization: nuclear p53 activates autophagy and cytosolic p53 inhibits autophagy (265). SIRT1 deacetylation of p53 blocks nuclear translocation during oxidative stress (266). ATG7, ATG8 and ATG5 have been shown to be direct targets for deacetylation by SIRT1 that function in the cytosol (189). Indeed, even a mutated SIRT1 restricted to the cytosol stimulated autophagy (261).

Acetylation is beginning to become a new area of research involved in canonical and non-canonical autophagy regulation (267;268). Specifically, mitochondrial acetylation is involved in the mitophagy mechanism (269). Knock out of GCN5L, a mitochondrial acetyl-transferase, diminishes mitochondrial protein acetylation and accumulates LC3 and p62 onto the mitochondria for canonical mitophagy independent of Parkin (269;270). GCN5L KO cells are able to sustain mitochondrial contain through
PGC-1α transcription, but enhance mitophagy by activating TFEB in a manner independent of mTOR (271). While the involvement of SIRT1 within this pathway is unknown, several studies support that SIRT1 may have a role in regulating this pathway. SIRT1 regulates cellular mitochondrial content is at least in part due to the deacetylation of PGC-1α by increasing the transcription of genes involved in mitochondrial biogenesis (193;194;196;197), and autophagy (190;272-274). Additionally, within the nucleus, GCN5 counteracts SIRT1 deacetylation of PGC-1α in neurons (275;275). While the role of GCN5L and SIRT1 induced mitophagy is unknown, it appears to be an exciting new area for future studies.

Despite the extra mitochondrial localization of SIRT1, some reports have indicated that SIRT1 can localize within the mitochondria (196;276). Protein mitochondrial localization requires an N-terminal mitochondrial targeting sequences (MTS), and SIRT1 possessing an MTS has not been shown. However, SIRT1 may localize to the mitochondria through protein-protein interactions on the mitochondrial outer membrane. While roles of mitochondrial SIRT1 remain unknown, SIRT3 has been shown to be the global regulation for mitochondrial acetylation (210). However, SIRT3 is localized to the matrix and outer mitochondrial membrane proteins do not span across the inner and outer membranes suggesting that SIRT1 and other HDACs may regulate mitochondrial functions through outer membrane deacetylation events.

**SIRT1 And I/R Injury**

Multiple factors lead to I/R injury and targeting specific areas has been disappointing. The pleiotropic nature of SIRT1 could have beneficial effects and be used as a therapeutic target to suppress I/R injury. Indeed, studies show that
pharmacological, surgical and genetic approaches to stimulate SIRT1 mediated cytoprotection during I/R to the heart, lung, intestines, brain, and liver. As impaired autophagy is a major factor leading to I/R injury, one can speculate that SIRT1 may play a role in autophagy regulation during liver I/R injury. Indeed, the enhancement of SIRT1 expression in transgenic mice was cytoprotective to myocardial I/R due to its up regulation of mitochondrial antioxidants, and autophagy proteins (259). SIRT1 overexpression also enhanced autophagy by increasing lysosomal fusion, and formation of autophagosomes mediated by Rab7 expression (190). In the kidney, caloric restriction increased mitochondrial autophagy through SIRT1 mediated Bnip3 expression (260;277). In the liver, fasting enhances SIRT1 induced autophagy to provide cytoprotection against I/R injury (164). Even the benefits of ischemic preconditioning are mediated through SIRT1 (278). Autophagy plays an integral role in I/R injury, however how SIRT1 affects autophagy during liver I/R injury remains unknown.

**Therapeutic Potential Of SIRT1 And Liver I/R Injury**

Liver I/R injury is a multifactorial phenomenon that contributes to mortality and morbidity of patients after liver resection surgery. SIRT1 is an appealing candidate, since it’s been shown to regulate several cellular processes, such as the maintenance of energy homeostasis and cellular survival, which are also the main functions of autophagic degradation. Pharmacological activation of SIRT1 during liver resection surgery will provide a novel and unexplored avenue to treat I/R injury by up regulating endogenous protective mechanisms like autophagy.
Figure 1-2. Liver lobules have hepatic zonal differences within the liver. (A) Histological section of liver tissue to demonstrate the structure of liver lobules. (B) Animations of a liver lobule and the zonal differences of a hepatic cord for a structural concept.
Figure 1-3. Mitochondrial permeability transition pore opening during reperfusion leads to mitochondrial rupture and hepatocyte death. (A) The main components of the mitochondrial permeability transition pore (VDAC, ANT and Cyclophilin D) localized in the mitochondria. (B) Upon reperfusion, an influx of calcium triggers the MPT onset and ROS generation leading to hepatocyte death. (C) Electron micrographs comparing mitochondria before and after I/R in hepatocytes, and an animation of the opening of the MPT pore leading to the swelling and rupturing of the mitochondria.
Figure 1-4. Accumulation of calcium leads to the MPT onset during I/R. (A) Sodium hydrogen exchanger (NHE) and sodium potassium ATPase (NKA) are two proteins involved in calcium homeostasis within the plasma membrane to maintain cytosolic ion balance. (B) Ischemia leads to the accumulation of cytosolic calcium due to ATP depletion and inhibition of NKA leading to reversed activation of the sodium calcium exchanger (NCE). (C) Reperfusion leads to mitochondrial repolarization that triggers the mitochondrial calcium accumulation through the calcium uniporter (CU). (D) Calcium in the mitochondria activates cyclophilin D to open the MPT pore that in turn leads to influx of solutes into the matrix causing the mitochondria to depolarize, swell and rupture.
Figure 1-5. The three different types of autophagy are chaperone mediated autophagy, microautophagy and macroautophagy. (A) Chaperone mediated autophagy uses the HSC70 complex or HSP90 to recognize misfolded proteins using the KFERQ sequence. The complexes localize and translocate a misfolded protein into the lysosome for degradation. (B) Microautophagy is the invagination of the lysosomal membrane for degradation. (C) Macroautophagy is a sequential process that begins with the initiation signal that elongates the phagophores membrane to form a mature autophagosome. Autophagosomes carrying cargo fuse with lysosomes to form an autolysosome that degrades proteins, and organelles for metabolite release back into the cytosol.
Autophagy initiation can occur through mTOR inhibition and AMPK activation. Activation of mTOR from Akt phosphorylation inhibits the activation of the ULK1/2 complex and autophagy initiation. Nutrient and amino acid deprivation leads to mTOR inhibition and ULK1/2 activation that can (1) phosphorylate Beclin1 to release Bcl-2 family members which promote autophagy and insert phosphatidylinositol 3 phosphate (PI3P) into the autophagosomal membrane, and (2) localize to the phagophore to recruit the autophagy elongation protein machinery. Furthermore, inhibition of mTOR leads to the dephosphorylation of transcription factor EB for lysosomal and autophagy protein generation through gene expression. Another mechanism for autophagy induction is through adenosine monophosphate kinase (AMPK). AMPK senses changing ATP levels within the cell by monitoring cAMP levels. AMPK activation can lead to direct phosphorylation of Beclin 1, ULK1/2 complex, and mTOR to stimulate autophagy. Other factors that have been shown to play a role in autophagy are proteins that can bind to the Beclin1-PI3KIII complex to drive or impair PI3P insertion into the autophagosomal membrane.
Figure 1-7. The ATG8 and ATG12 ubiquitin like systems process ProLC3 to LC3-II for insertion into the autophagosomal membrane. (A) The ATG8 system begins with the cleavage of an arginine residue of ProLC3 to generate LC3-I, which binds to ATG7 through a thioester bond that requires the hydrolysis of ATP. ATG7 is an E1 like ubiquitin activating enzymes in both the ATG8 and ATG12 systems. LC3-I is transferred to ATG3, which is the E2 ubiquitin like conjugating enzyme through another thioester bond. (B) The ATG12 system utilizes ATG10 as the E2 ubiquitin like conjugating enzyme through another thioester bond. Next, ATG12 is bound to ATG5 through an isopeptide bond to create the E3 ubiquitin like ligase for the insertion of LC-I onto the autophagosomal membrane to create an LC3-II protein that contains a phosphotidylethanolamine (PE). ATG16 oligomers bind with the ATG5-ATG12 complex to facilitate autophagosomal membrane ligation.
Figure 1-8. SIRT1 requires NAD$^+$ as a substrate for enzymatic activity. (A) Acetylated substrate is bound to SIRT1 that induces a conformational change that facilitates NAD$^+$ binding. (B) SIRT1 catalyzes NAD$^+$ and an acetylated protein to produce nicotinamide, a deacetylated protein, and O-Acetyl-ADP-ribose (2′-OAADPr).
Sirtuin 1 Interactions, Modifications and Domains

Figure 1-9. SIRT1 regulation can occur through post translational modifications and endogenous protein interactions. The SIRT1 amino acid sequence for SIRT1 includes two nuclear localization sequences (NLS), two nuclear export sequences (NES), and a conserved catalytic domain. Activator of SIRT1 (AROS) and Depletion in Breast Cancer 1 (DBC-1) are endogenous regulators of SIRT1 that can bind directly to the SIRT1 protein at the indicated sites. Post translational modifications can regulate SIRT1 function and the individual amino acids involved in both the human and mouse sequences are listed.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Volume (Percent)</th>
<th>Cell number (Percent)</th>
<th>Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
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<td>80</td>
<td>60-65</td>
<td>Hepatic Cords</td>
<td>Main metabolic cell</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Absorption and secretion</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Macromolecule synthesis</td>
</tr>
<tr>
<td>Non Hepatocytes</td>
<td>6.3</td>
<td>30-40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial</td>
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<td>15-20</td>
<td>Per sinusoidal</td>
<td>Fluid and material exchange</td>
</tr>
<tr>
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<td>8-12</td>
<td>Sinus endothelium</td>
<td>Phagocytosis, Discharge of signal substances,</td>
</tr>
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<td>1.4</td>
<td>3-8</td>
<td>Space of Disse</td>
<td>Synthesis extracellular matrix,</td>
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<td>and sinus endothelium width</td>
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<tr>
<td>PIT</td>
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<td>0.1</td>
<td>Sinusoids and</td>
<td>Remove foreign cells</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Space of Disse</td>
<td></td>
</tr>
<tr>
<td>Biliary Epithelial</td>
<td>3.5</td>
<td></td>
<td>Biliary ducts</td>
<td>Biligenesis</td>
</tr>
</tbody>
</table>

**Table 1-1. Liver composition by cell type**
Table 1-2. Hepatic zones have structural and functional differences within a liver lobule.

<table>
<thead>
<tr>
<th>Zone 1</th>
<th>Zone 3</th>
</tr>
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<tbody>
<tr>
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<td>Hepatocytes</td>
</tr>
<tr>
<td>Size</td>
<td>Size</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>Large</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Large</td>
</tr>
<tr>
<td>Small</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Golgi membrane</td>
<td>Large</td>
</tr>
<tr>
<td>Smooth ER</td>
<td>Large</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>Small</td>
</tr>
<tr>
<td>Non Hepatocyte</td>
<td>Endothelial</td>
</tr>
<tr>
<td>Endothelial fenestration</td>
<td>Endothelial fenestration</td>
</tr>
<tr>
<td>Kupffer cell number</td>
<td>Kupffer cell number</td>
</tr>
<tr>
<td>Ito cell number</td>
<td>Ito cell number</td>
</tr>
<tr>
<td>PIT cells</td>
<td>PIT cells</td>
</tr>
</tbody>
</table>

Zonal Functions

- Gluconeogenesis
- Fatty acid oxidation
- Urea synthesis
- Glutamine hydrolysis
- Amino acid degradation
- Bile acid – dependent fraction
- Glycogen synthesis from lactate and amino acids
- Glycogen degradation
- Cholesterol synthesis
- Citrate cycle
- Respiration chain reactions
- Pigment deposition

- Glycolysis
- Liponeogenesis
- Glutamine synthesis
- Glutamate transport
- Bile acid – independent fraction
- Glycogen synthesis from glucose
- Glycogen degradation from lactate
- Biotransformation
- Ketogenesis

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Localization</th>
<th>Activity</th>
<th>Targets</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT1</td>
<td>Cytosol, Nucleus</td>
<td>Deacetylation</td>
<td>FOXO1, FOXO3A, p53, PGC-1α, ATG7, ATG12, ATG8, SREBP-1c, CREB, Ku70, NF-κB, LXR, and more</td>
<td>Autophagy, Transcription, Mitochondrial Biogenesis, Cell cycle</td>
</tr>
<tr>
<td>SIRT2</td>
<td>Cytosol</td>
<td>Deacetylation</td>
<td>FoxO1, PEPCK, PAR3</td>
<td>Cell cycle, Autophagy Tumorigenesis</td>
</tr>
<tr>
<td>SIRT3</td>
<td>Cytosol, Nucleus, Mitochondria</td>
<td>Deacetylation</td>
<td>Cyclophilin D, LCAD, GDH, IDH2, Complex I, and more</td>
<td>Bioenergetics Autophagy, MPT onset</td>
</tr>
<tr>
<td>SIRT4</td>
<td>Mitochondria</td>
<td>ADP-Ribosylation</td>
<td>GDH</td>
<td>Insulin secretion</td>
</tr>
<tr>
<td>SIRT5</td>
<td>Mitochondria</td>
<td>Deacetylation, Demalonylation, Desuccinylation, Deglutylation</td>
<td>CPS1</td>
<td>Urea cycle</td>
</tr>
<tr>
<td>SIRT6</td>
<td>Nucleus</td>
<td>Deacetylation, ADP-ribosylation</td>
<td>H3K9, H3K56</td>
<td>DNA repair</td>
</tr>
<tr>
<td>SIRT7</td>
<td>Nucleus</td>
<td>unknown</td>
<td>Unknown</td>
<td>rDNA transcription</td>
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</table>
CHAPTER 2
LIVER ISCHEMIA/REPERFUSION CAUSES THE DEPLETION OF SIRTUIN 1 AND HEPATOCELLULAR DEATH

Introduction

Hepatectomy is the operative removal of the liver, which can be partial for a resection or more 90% for major liver surgery. Partial and major liver surgery often subjects the liver to ischemia/reperfusion (I/R) due to vasculature clamping. In addition, the explant of the donor liver during transplantation encounters a severe I/R injury. Liver I/R injury is a major factor leading to liver dysfunction and patient mortality after surgery. Current therapeutics and surgical approaches to circumvent I/R injury are finite or ineffective. Sirtuins are class III histone deacetylases with non-histone targets. Sirtuin 1 (SIRT1) has been proposed to mediate cytoprotection against nonhepatic I/R injury in mice and rats, however the role of SIRT1 during liver I/R injury remains unclear. We hypothesize that the loss of SIRT1 during liver I/R is a contributing factor leading to hepatocyte death. Human tissue was collected from the transection margin before and after 15 minutes of inflow occlusion during a partial hepatectomy. Immunoblot analysis was used to determine SIRT1 expression. SIRT1 significantly reduced in tissue that underwent inflow occlusion. Next, we confirmed these changes in mouse livers and hepatocytes subjected to I/R. Furthermore, prolonged ischemia leads to the loss of SIRT1, which correlated with I/R induced hepatocyte death. Attempting to suppress the reduction, we identified that SIRT1 is depleted in a multifactorial manner during I/R, which involves cathepsins and calpains. Interestingly, partial suppression of SIRT1 through calpain inhibition suppressed hepatocyte death. These data suggest that the depletion of SIRT1 leads to hepatocyte death and SIRT1 may provide cytoprotection against liver I/R injury.
Background

Liver resection, or partial hepatectomy, is described as the operative removal of a segment of the liver. Liver resections are performed as a treatment for various liver diseases including cancer, benign tumors and cystic disease. In addition, liver transplant patients undergo a complete removal of the liver prior to donor implantation. During liver surgeries, no-flow ischemia is often utilized to minimize intraoperative blood loss, which exposes the liver to an inevitable I/R event. Although prolonged ischemia eventually causes tissue injury, severe damage paradoxically does not occur until reinstitution of blood flow and return to normal physiological pH, an event called reperfusion injury (9;15;279;280). Current therapeutic approaches to circumvent I/R injury remain ineffective, thus new strategies are urgently required to suppress liver damage due to I/R injury.

Hepatocytes are the parenchymal cells of the liver and make up about 80% of the total liver by volume. During liver resection surgery, warm I/R is the causative factor leading to hepatocyte damage and post-operative liver failure (32;34;281). Parenchymal cell damage leads to increased patient morbidity and mortality following liver surgery (27). The mechanisms underlying I/R injury are multifactorial with mitochondrial dysfunction as the major contributor to tissue death after I/R (59). Upon reperfusion, the unregulated opening of the mitochondrial permeability transition (MPT) pores leads to mitochondrial dysfunction and hepatocyte death.

Sirtuins are class III histone deacetylases that play an integral role in energy homeostasis, cell survival, longevity, and autophagy (189;190;259). Sirtuin localization and activity are specific for each of the seven different isoforms. Ischemic preconditioning and caloric restriction convey beneficial effects that are mediated
through SIRT1 to protect against liver I/R injury (164;278). SIRT1 is a pleiotropic protein that regulates mitochondrial biogenesis, oxidative phosphorylation, and autophagy (194;197). SIRT3, a mitochondrial matrix protein, regulates mitochondrial oxidative phosphorylation and reactive oxygen species generation (216;282-289). In myocytes, SIRT3 mediated cytoprotection against I/R injury by deacetylating cyclophilin D to block the unregulated opening of the MPT pores and prevents mitochondrial dysfunction (290). SIRT5 is a mitochondrial matrix protein that regulates the urea cycle through carbamoyl phosphate synthase (CPS1) (217;291). An accumulation of ammonia in the blood stream occurs during liver dysfunction leading to hepatic encephalopathy (292;293); however the role of SIRT5 during liver I/R injury remains to be elucidated. Sirtuins have been proposed as therapeutic targets for treating liver steatosis, alcoholic liver disease, obesity, and diabetes (194;203;294-296), however little information links sirtuin function with hepatic I/R injury.

The goal of the present investigation was to determine if hepatic I/R affects sirtuins. Sirtuin expression was altered in human liver tissue after inflow occlusion. Next, we established in vivo and in vitro models of liver I/R in a mouse and simulated I/R in primary hepatocytes that would mimic the changes in sirtuin expression observed in patients undergoing partial hepatectomy. SIRT1 was shown to decrease during ischemia leading to reperfusion induced hepatocyte death, thus we attempted to identify the reduction mechanism using inhibitors for the proteasome, cathepsins and calpains. The ischemic reduction of SIRT1 occurred through multiple proteases, but only calpain inhibition prevented the depletion of SIRT1 and hepatocyte death after prolonged ischemia followed by reperfusion. Collectively, this study supports that SIRT1 plays a
key role in liver I/R injury and the ischemic depletion of SIRT1 leads to hepatocyte death in a manner dependent on calpains.

Materials And Methods

Human Liver Tissue Collection

All human tissue was collected, stored and treated according to protocols approved by the Institutional Review Board at the University of Florida. Liver tissue from the transection margin was collected before and after 15 minutes of inflow occlusions and immediately flash frozen in liquid nitrogen. Immediately, tissue was homogenized on ice in radioimmunoprecipitation buffer (RIPA) for immunoblot with protease and phosphatase inhibitors.

Mouse Liver In Vivo I/R

All animals received humane care according to protocols approved by the Institutional Care and Use Committee of the University of Florida. Hepatic inflow occlusion was performed by clamping the portal triad for 45 minutes. Reperfusion was initiated by removing a microvascular clamp (297). Liver biopsies from the left lateral lobe were collected during I/R and homogenized in RIPA buffer in the presence of protease and phosphatase inhibitors.

Tamoxifen Treatment For Inducible SIRT1 KO Mice

SIRT1 KO mice have a C57 BL/6 background and harbor a Cre-ERT2 fusion protein consisting of Cre recombinase fused to a triple mutant form of the human estrogen receptor; created in Dr. Sinclair’s laboratory at Harvard University (298). To induce SIRT1 knockout, 3-4 month old male mice containing a Cre-ERT2 fusion protein were injected intraperitoneally with 100 µl of Tamoxifen dissolved in a sterile corn oil (40 mg/ml) at Day 0 and Day 3.
Genotyping And RT-PCR

For genotyping, an ear punch or tail snip was digested in Buffer A (50 mM NaOH, 0.2 mM EDTA) for 30-60 minutes at 95°C followed by a brief vortex. The sample was incubated at room temperature for 5 minutes prior to the addition of 100 μL of buffer B (1M Tris pH 8.0). Samples were then centrifuged at 15,000 x g for 2 minutes to collect the debris followed by extraction of 2 μL of the supernatant for RT-PCR.

mRNA was extracted from 1 x 10^6 hepatocytes using 500 μl Trizol (Invitrogen, Carlsbad, CA) followed by isopropanol precipitation. mRNA concentrations and purity were measured using an Eon spectrophotometer (Biotek instruments, Winooski, VT) with a Take3 micro-volume plate (Biotek instruments, Winooski, VT). cDNA was synthesized using 2 μg mRNA using the Invitrogen SuperScript III First-Strand (Carlsbad, CA cat# 18080-051) using the random hexamer option.

For RT-PCR, cDNA (2 μL) was used with 1 μL of 100 nM primers for SIRT1-Forward (5’-GCCCATTTAAAGCAGTATGTG’3), SIRT1-Reverse (5’-CATGTAATCTCAACCTTGGAG’3), β-actin-Forward (5’-GTGGGCCGCTCTAGGCACCAA’3), and β-actin-Reverse (5’-CTCTTTGATGTACGCACGATTTCC’3). PCR was carried out using New England Bio Labs One Taq DNA Polymerase system (Ipswich, MA) using the Eppendorf MasterCycler 5333 Version 2 (Hamburg, Germany). cDNA was denatured for at 95°C for 5 minutes followed by 32 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute.
Hepatocyte Isolation And Culture

Hepatocytes were isolated from 3 month old male C57BL/6 mice by collagenase perfusion method. Mice were given an intraperitoneal injection of ketamine (100-200 mg/kg) and xylazine (10-20 mg/kg). The abdominal cavity was opened followed by inferior vena cava cannulation. Next, the portal vein was cut to allow the liver to decompress during perfusion. Finally, the suprahepatic vena cava was clamped to prevent flow to the heart. The liver was perfused with Buffer A (25 mmol/L HEPES, 115 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L MgSO$_4$, 1 mmol/L KH$_2$PO$_4$, 0.5 mmol/L EGTA, 2 mmol/L MgSO$_4$) for 5 minutes prior to Buffer B (25 mmol/L HEPES, 115 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L KH$_2$PO$_4$, 0.09 mg/ml collagenase) perfusion for 6-8 minutes at a flow rate of 4 ml/min. The last 2 minutes of perfusion with Buffer B was accompanied by a gentle massage to the liver. The liver was removed and hepatocytes were extracted by a gentle agitation in Buffer C (25 mmol/L HEPES, 115 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L MgSO$_4$, 1 mmol/L KH$_2$PO$_4$, 10% Bovine Serum Albumin). After sequential centrifugation for cell purification, hepatocytes were seeded on precoated dishes and/or plates with 0.1% type 1 rat tail collagen. Only hepatocytes with viability of greater than 90%, as judged by trypan blue exclusion after isolation were used. Isolated hepatocytes were cultured in Waymouth medium MB-752/1 containing 2 mmol/L L-glutamine, 27 mmol/L NaHCO$_3$, 10% fetal calf serum, 100 nmol/L insulin, and 100 nmol/L dexamethasone. After 4 hours, isolated hepatocyte media was changed to remove dead and non-adhered hepatocytes.

Hepatocyte Simulated I/R

Hepatocytes were incubated at 37°C in Krebs–Ringer–HEPES (KRH) buffer (25 mmol/L HEPES, 115 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L MgSO$_4$, 1 mmol/L KH$_2$PO$_4$, 10 mmol/L Na$_2$HPO$_4$, 10 mmol/L NaCl, 1 mmol/L MgSO$_4$, and 20 mmol/L KCl) for 4 hours. The media was then changed to new KRH buffer containing 27 mmol/L NaHCO$_3$, 10% fetal calf serum, 100 nmol/L insulin, and 100 nmol/L dexamethasone.
KH$_2$PO$_4$) at pH 6.2 in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). To simulate reoxygenation and return to physiologic pH during reperfusion, anaerobic KRH at pH 6.2 was replaced with aerobic KRH at pH 7.4 (50;297;299).

**Reagents And Drug Treatments**

E64d was purchased from Enzolife (Farmdale, NY, BML-PL107). MG-132 was purchased from Fischer Scientific (Waltham, MA), and Acetyl-Leucine-Leucine-Methionine (ALLM) was purchased from Calbiochem (Darmstadt, Germany). Cells were treated with E64d and MG-132 for 1 hour before hepatocytes underwent I/R, while ALLM treatment was 12 hour before I/R. All inhibitors were continuously present during I/R. All other chemicals were of analytical grade and obtained from Sigma Aldrich (St Louis, MO).

**Immunoblot**

Lysates of hepatocytes were obtained using RIPA buffer (25 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 1% Deoxycholate, 5 mM EDTA, 0.1% SDS containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Tissue and whole cell lysate were used to analyze protein expression unless indicated. Antibodies against β-actin were purchased from Sigma Chemical Co (St Louis, MO). The SIRT1 antibody was purchased from Millipore (Temecula, CA). The SIRT3 antibody for human samples was purchased from Cell Signaling Technology (Danvers, MA), while the mouse SIRT3 antibody was purchased from Santa Cruz (Santa Cruz, CA). SIRT5 was purchased from Abcam (Cambridge, MA). Changes in protein expression were determined using Image J software (National Institutes of Health, Bethesda, MD).
Cytosolic And Nuclear Subfractionation

Primary hepatocytes were subfractionated using Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents (Rockford, IL) as the manufacturer recommended.

Cell Death Assay Using Propidium Iodide

Hepatocytes were seeded in a 24 well plate (Falcon, Lincoln Park, NJ) at 1.5 x 10^5 cells per well and incubated overnight in Waymouth media. Cells were incubated with 30 µM propidium iodide (Sigma Aldrich, St Louis, MO, P4170) in KRH pH 7.4 for 5 minutes prior to I/R. Cells were then incubated in 30 µM propidium iodide in KRH pH 6.2 during ischemia followed by reperfusion in 30 µM propidium iodide in KRH pH 7.4. Cell death was determined after 2 hours of reperfusion by the addition of 20 µM digitonin (Sigma Aldrich, St Louis, MO, D5628). Necrosis at 5, 60, and 120 minutes of reperfusion was determined using propidium iodide (50;53;57).

Data Analysis

Differences between groups were compared using analysis of variance and post hoc Bonferroni analysis (SigmaStat, Ashburn, VA). P<0.05 denotes statistical significance. Data are expressed as means ± SE. All experiments are representative of at least 3 different cell isolations or animals per group.

Results

Ischemia Causes SIRT1 Depletion

To investigate the changes in sirtuins after ischemia, human liver biopsies were collected before and after inflow occlusion-induced ischemia. Ischemia alone decreased SIRT1 levels to 28 ± 15% of the control, while mitochondrial SIRT3 and SIRT5 did not decrease in human liver tissue (Figure 2-1A). To confirm these changes, mouse livers
were subjected to *in vivo* I/R. Similar to the human samples, ischemia led to a significant reduction in SIRT1 that was not restored during reperfusion, SIRT3 remained constant and SIRT5 levels increased after I/R (Figure 2-1B).

To determine if these changes were occurring in the parenchymal cells of the liver, isolated hepatocytes were subjected to simulated I/R to analyze SIRT1, SIRT3 and SIRT5 expression. SIRT1 decreased progressively during ischemia and was undetectable after 4 hours, while SIRT3 and SIRT5 increased during reperfusion after 4 hours of ischemia (Figure 2-1C). Propidium iodide fluorometry showed that substantial necrosis during reperfusion occurred only after prolonged ischemia (Figure 2-2A). To correlate SIRT1 expression with cell death, SIRT1 expression was determined with various periods of ischemia. Shorter ischemic times followed by reperfusion showed that ischemia reduced SIRT1. However, similar levels of SIRT1 were observed after reperfusion and hepatocytes remained viable (Figure 2-2B). SIRT1 localizes to the cytosol and nucleus (185), therefore we analyzed changes in SIRT1 expression in the cytosolic and nuclear fractions during I/R. After 2 hours of ischemia, cytosolic SIRT1 was rapidly degraded, while nuclear SIRT1 did not decrease until 4 hours of ischemia (Figure 2-2C). Taken together these results show that I/R depletes SIRT1, but not mitochondrial SIRT3 or SIRT5 in livers and hepatocytes.

To further clarify an integral role of SIRT1 in I/R injury, hepatocytes were isolated from Tamoxifen-inducible SIRT1 knockout mice (SIRT1 KO) (a generous gift from Dr. Sinclair) (298) and subjected to different times of I/R. While the hepatocytes from wild type (WT) mice well tolerated 2 hours of ischemia and reperfusion, the cells from SIRT1 KO mice displayed an increase in reperfusion injury (Figure 2-3), showing a heightened
vulnerability of SIRT1-null cells to sublethal I/R conditions. This observation was, indeed, anticipated because WT cells after 4 hours of ischemia were eventually devoid of SIRT1. Taken together, these studies firmly support the hypothesis that the loss of SIRT1 contributes to hepatic I/R injury.

**SIRT1 Depletion Is Multifactorial**

To investigate the mechanisms underlying SIRT1 depletion after I/R, changes in SIRT1 mRNA were measured with PCR. As shown in Figure 2-4A, I/R did not alter SIRT1 mRNA, suggesting that SIRT1 depletion is a post-translational process. Using cycloheximide (CHX), a protein synthesis inhibitor, we estimated the half-life of SIRT1 to be about 14 hours (Figure 2-4B), which suggesting that SIRT1 depletion is likely to be associated with the ischemic stress. To identify the factor(s) involved in SIRT1 depletion, hepatocytes were treated with inhibitors for calpain, cathepsin, and the proteasome and analyzed by immunoblot. Calpain activation leads to hepatic I/R injury(51;53), Acetyl-Leu-Leu-Met (ALLM), a calpain inhibitor, partially suppressed the loss of SIRT1 (Figure 2-5A) but prevented I/R injury (Figure 2-5B). These results suggested that calpain activation during I/R is, at least in part, responsible for SIRT1 depletion. An integral role of SIRT1 was confirmed by a loss in ALLM induced cytoprotection against I/R in SIRT1 KO hepatocytes (Figure 2-5C). MG-132, a proteasome inhibitor, did not suppress the depletion of SIRT1 or cell death after reperfusion (Figure 2-6). Cathepsins have been reported to degrade SIRT1 in endothelial cells (300). Cathepsins are a family of proteases found mostly within the lysosome, which have a role in the cleavage of SIRT1 (300;301). Lysosomal leakage during oxidative stress and I/R can cause the releases of these hydrolytic enzymes into the cytosol (78-80). E64d, a pan cysteine protease cathepsin inhibitor, partly
suppressed the SIRT1 reduction after 2 hours of ischemia (Figure 2-7A). Neutralization of the lysosome using chloroquine indicates that cathepsin proteolysis of SIRT1 is independent of lysosomal degradation (Figure 2-7B). After 4 hours of ischemia and reperfusion, cathepsin inhibition did not affect the depletion of SIRT1 or cell death (Figure 2-7C). Furthermore, the administration of both ALLM and E64d showed minor additive effects on SIRT1 depletion and necrosis (Figure 2-8). Collectively, these data indicate that the depletion of SIRT1 during I/R is caused by multiple factors involving calpains and other proteases.

**Discussion**

Liver I/R injury remains a fundamental complication during surgery leading to liver failure and patient mortality (13). The current ineffectiveness of therapeutics to ameliorate liver I/R injury prompts the investigation into new potential targets to provide protection against I/R injury. Here we demonstrate that (a) ischemia decreases SIRT1 in human and mouse livers, and primary hepatocytes, (b) the loss of SIRT1 sensitizes hepatocytes to reperfusion induced death, (c) calpain activation leads to the depletion of SIRT1, and (d) calpain inhibition prevents SIRT1 depletion and suppresses hepatic I/R injury (Figure 2-9). Overall, this study shows that the loss of SIRT1 is associated with hepatocyte death during liver I/R injury.

Sirtuins are potential therapeutic targets for several different liver diseases. SIRT1, the most studied sirtuin family member, is a pleiotropic protein involved in mitochondrial metabolism and autophagy (190;260;298;302;303). SIRT3 is purposed to regulate energy metabolism through oxidative phosphorylation (216;282;284;289;304;305), while SIRT5 is one of the least studied sirtuins and shown to have a critical role in the mitochondrial urea cycle (217;291). SIRT1 and SIRT3 have
a cytoprotective role against I/R injury in myocytes (259;290), however little is known about roles of SIRT1, SIRT3 and SIRT5 during hepatic I/R injury. To our knowledge, we are the first to report SIRT1, SIRT3 and SIRT5 expression in human liver tissue after 15 minutes of inflow occlusion-induced ischemia. In human tissue, ischemia caused the reduction of SIRT1 with no alteration to SIRT3, and an elevation in SIRT5 (Figure 2-1). Our mouse model for in vivo liver I/R responded in a similar manner that resembled the human inflow occlusion induced ischemia (Figure 2-1). Our primary hepatocyte model also resembled human and mouse liver I/R injury as indicated by a reduction in SIRT1 and an elevation in SIRT5, however SIRT3 became further elevated in this system. This unusual SIRT3 response requires further investigation but may be caused by the loss of temporal and spatial factors found in liver, which are absence in the isolated hepatocyte system.

Ischemic preconditioning and caloric restriction convey SIRT1 mediated cytoprotection against I/R injury to steatotic livers in rats and mice (164;278). Nutrient starvation activates SIRT1 (195) and suppresses reperfusion induced death after I/R in hepatocytes (47). These studies suggest a correlation with SIRT1 conveying cytoprotection against I/R injury. To further support this, hepatocytes undergoing prolonged ischemia lose SIRT1 and following reperfusion leads to cell death (Figure 2-2). Prolonged ischemia and hepatocyte death are causative factors leading to post liver resection failure (32-35). Consistent with previous studies (39;50;58), hepatocytes subjected to 4 hours of ischemia led to reperfusion induced death at 60 minutes, which occurred in cells that were depleted of SIRT1. Reperfusion after ischemia for 1 and 2 hours maintained hepatocellular viability. Furthermore, a significant reduction of SIRT1
during ischemia was sustained during reperfusion (Figure 2-2). After 4 hours of ischemia, SIRT1 was depleted and hepatocytes underwent reperfusion induced death, while SIRT5 expression was heightened. An elevation in SIRT5 has been suggested to up regulate CPS1 and mitochondrial urea clearance (217;291), however the role of SIRT5 during liver I/R remains unclear.

SIRT1 localizes to both the cytosol and nucleus, however only the nuclear SIRT1 was able to suppress oxidative stress (185). In hepatocytes, cytosolic SIRT1 is reduced during 1 and 2 hours ischemia while nuclear SIRT1 remained unchanged up to 2 hours of ischemia prior to depletion (Figure 2-2). These data suggest that cytosolic SIRT1 reduction precedes nuclear SIRT1 loss in hepatocytes before depletion. To establish that the loss of SIRT1 leads to hepatocyte death, SIRT1 KO hepatocytes were subjected to 2 hours of ischemia and reperfusion, which are sublethal I/R conditions. SIRT1 null hepatocytes were significantly more sensitive to sublethal I/R, in comparison to wild type cells (Figure 2-3). Collectively, these studies firmly indicate that SIRT1 plays an integral role in liver I/R injury.

Mechanisms involving protein degradation and cleavage during I/R has been a controversial topic for over 30 decades (62;306). Ischemia leads to protein damage and degradation of a large number of intracellular proteins (62). Based on our finding, ischemia leads to the reduction of SIRT1 at the protein level without affecting mRNA expression (Figure 2-4), which differs from an in vivo heart model of I/R where SIRT1 mRNA levels decreased (259). SIRT1 chemical stability and normoxic protein turnover could not account for the rapid deletion of SIRT1 during 4 hours of ischemia (Figure 2-4), suggesting that ischemia leads to the degradation of SIRT1. SIRT1 contains several
cysteine residues (Cys$^{67,68,482,490}$) that are sensitive to redox stress and its modification can lead to degradation through the proteasome (307). The ubiquitin-proteasomal system (UPS) is a non lysosomal dependent degradation mechanism to remove proteins using ubiquitin as a post translational signal for degradation. Degradation of SIRT1 in endothelial cells, chondrocytes and adipocytes occurs through the proteasome (300;301;308). During I/R, a functional UPS response remains controversial, some studies showed that ATP depletion blocks ubiquitin protein ligase and proteasomal assembly, stability, and functions, while others demonstrated an increase in protein ubiquitination and sustained proteasomal activity (309). We found that inhibition of the proteasome did not suppress the reduction of SIRT1 in hepatocytes during I/R (Figure 2-6).

Prior to proteasomal degradation, cathepsins, a family of proteases found mostly within the lysosome, have been implicated in the cleavage of SIRT1 (300;301). There are over a dozen different cathepsin members that become activated by low pH, however the cysteine cathepsins B and D are stable and retain some activity under neutral pH (76;77). In chondrocytes, cathepsin B cleaves SIRT1 after tumor necrosis factor alpha (TNF-α) treatment (301). After 2 hours of ischemia, the pan cysteine cathepsin inhibitor suppressed the degradation of SIRT1, while lysosomal neutralization had no effect (Figure 2-7). The increase in SIRT1 expression by cathepsin inhibition but not lysosomal neutralization may suggest that cathepsins are being translocated from lysosomes to the cytosol during ischemia, as proposed by previous reports (72;78-80;84). Lysosomal rupture has been proposed as an event that precedes the MPT onset leading to cell death (47) and hepatocytes remain viable at 2 hours of ischemia
implicating that the lysosomes did not rupture but may be releasing cathepsins into the cytosol. Further investigations are required to characterize lysosomal instability during liver I/R injury. Pharmacological inhibition of cathepsins has been reported to reduce cerebral I/R induced cell damage (67;82;83), however cathepsin inhibition did not suppress hepatocyte death or the loss of SIRT1 after prolonged ischemia followed by reperfusion (Figure 2-7) indicating that SIRT1 degradation is multifactorial.

Calpains are a family of non lysosomal cysteine proteases that degrade intracellular proteins. During I/R, calpains become active and play a crucial role in reperfusion induced cell death (51;53) Consistent with our previous finding (51-53), calpain inhibition suppressed hepatocyte death during I/R (Figure 2-5). Calpain inhibition, however, did not suppress the reduction of SIRT1 during 2 hours of ischemia (Figure 2-5). Prolonging ischemia increases calpain activity (52) and at 4 hours of ischemia calpain inhibition prevented SIRT1 depletion suggesting calpains play a partial role in the loss of SIRT1 during ischemia. Furthermore, calpain inhibition induced cytoprotection against I/R injury was mediated by SIRT1 as indicated by the loss of cytoprotection in SIRT1 KO hepatocytes (Figure 2-5). As a note, inhibition of both calpain and cathepsin only slightly increased SIRT1 expression and hepatocyte survival suggesting other factors are still involved in the SIRT1 reduction mechanism (Figure 2-8). Other possible targets to suppress the reduction of SIRT1 during ischemia are (a) matrix metalloproteinases (MMPs), (b) aspartyl cathepsin proteases and (c) serine cathepsin proteases. MMPs have shown to cleave intracellular substrates during I/R (310). However the role of MMPs in SIRT1 degradation has yet to be explored.
Furthermore, since aspartyl and serine proteases would not have been inhibited by E64d, the potential roles of these proteases cannot be excluded.

In conclusion, we show that SIRT1 may have a cytoprotective role during liver I/R injury and the loss of SIRT1 sensitizes hepatocytes to I/R injury. Ischemia leads to the proteolysis of SIRT1 in a multifactorial manner. Cathepsins can initially cleavage SIRT1 during short ischemic times but hepatocytes remain viable under this condition. Prolonging ischemia leads to activation of calpains that depletes SIRT1 leading to reperfusion induced death.
Figure 2-1. SIRT1, SIRT3, and SIRT5 expression changes in human and mouse livers and primary hepatocytes subjected to I/R. (A) Immunoblot analysis of SIRT1, SIRT3 and SIRT5 expression in human liver tissue after 15 minutes of inflow occlusion induced ischemia. (n=3) (B) Immunoblot analysis of SIRT1, SIRT3 and SIRT5 mouse livers during in vivo I/R at indicated times. (n=3). (C) Immunoblot analysis of SIRT1, SIRT3 and SIRT5 in mouse primary hepatocytes subjected to simulated I/R at indicated times. *, p<0.05, **, p<0.01, and ***, p<0.001

Figure 2-2. Prolonged ischemia depletes SIRT1 leading to hepatocyte death during reperfusion. (A) Hepatocyte death was measured using 30 μM propidium iodide after 1, 2 and 4 hours of ischemia followed by reperfusion at the indicated times (n=5). (B) Immunoblot analysis of SIRT1 from hepatocytes undergoing various I/R conditions (n=3). (C) Immunoblot analysis of SIRT1 localization in the cytosolic (C) and nuclear (N) fractions during ischemia (n=3). *, p<0.05**, p<0.01, and ***, p<0.001
Figure 2-3. SIRT1 KO sensitizes hepatocytes to I/R injury. (A) Mouse tail snip and hepatocyte mRNA were analyzed to confirm SIRT1 KO. (B) Wild type (WT) and SIRT1 KO hepatocytes were subjected to various times of I/R to determine cell death using propidium iodide fluorometry (n=3)**, p<0.01

Figure 2-4. SIRT1 mRNA does not decrease during I/R and the protein is stable at 4 hours after protein synthesis inhibition using cycloheximide. (A) Hepatocytes were subjected to I/R and mRNA was collected for gross analysis of SIRT1 mRNA using RT-PCR. (n=3) (B) Hepatocytes treated with 35 μM CHX were analyzed for changes SIRT1 expression at indicated times using immunoblot analysis. (n=3)
Figure 2-5. SIRT1 mediates ALLM induced cytoprotection against I/R injury. (A) Immunoblot analysis of SIRT1 expression from hepatocytes treated with ALLM for 12 hours prior to and continuously during I/R. (n=3). (B) WT and (C) SIRT1 KO hepatocytes treated with 10 μM ALLM or DMSO for 16 hours before and continuously during I/R to measure reperfusion induced death after 4 hours of ischemia (n=3). *, p<0.05 and **, p<0.01

Figure 2-6. Proteasome inhibition using MG-132 does not suppress the ischemic reduction of SIRT1. (A) Immunoblot analysis of SIRT1 expression from hepatocytes treated with MG-132 for 1 hour prior to and continuously during I/R. (n=3). (C) Hepatocytes treated with different doses of MG-132 for 1 hour before and continuously during I/R to measure reperfusion induced death after 4 hours of ischemia (n=3).
Figure 2-7. Cathepsin inhibition using E64d suppresses the SIRT1 reduction after 2 hours of ischemia. (A) Immunoblot analysis of SIRT1 expression from hepatocytes treated with E64d for 1 hour prior to and continuously during I/R. (n=3). (B) Hepatocytes treated with 50 μM E64d or 10 μM CQ were subjected to various I/R conditions to analyze SIRT1 expression. Cells were treated with CQ for 1 hour prior to and continuously during I/R. (n=2) (C) Hepatocytes treated with different doses of E64d for 1 hour before and continuously during I/R to measure reperfusion induced death after 4 hours of ischemia (n=3).* p<0.05

Figure 2-8. Cathepsin and calpain combined inhibition does not have an additive effect on suppressing the reduction of SIRT1 during I/R. (A) Immunoblot analysis of SIRT1 expression from hepatocytes treated with 10 μM ALLM for 16 hours and 50 μM E64d for 1 hour prior to and continuously during I/R. (n=3). (B) Hepatocytes treated with ALLM and E64d were used to measure reperfusion induced death after 4 hours of ischemia (n=3).* p<0.05, and ** p<0.01
Figure 2-9. The ischemic reduction of SIRT1 is multifactorial involving cathepsins, calpains and other proteases. Graphical interpretation of the mechanism for SIRT1 depletion during I/R. Cysteine cathepsin proteases hydrolyze SIRT1 during short ischemic times, which does not lead to reperfusion induced death. Prolonging ischemia leads to calpain activation and SIRT1 depletion leading to reperfusion induced death.
CHAPTER 3
SIRTUIN 1 ACTIVATION PREVENTS MITOCHONDRIAL DYSFUNCTION AND PROMOTES AUTOPHAGY TO PROTECT AGAINST LIVER I/R INJURY

Introduction

Chapter 2 described the ischemic reduction of Sirtuin 1 (SIRT1) caused by multiple proteases leading to hepatocyte death during ischemia/reperfusion (I/R) injury. In this chapter, we investigate whether modulating SIRT1 expression and activity influences mitochondrial dysfunction, autophagy and cell death during I/R. Autophagy is a cytoprotective mechanism to suppress hepatocyte damage during I/R injury (51-53). SIRT1 mediates autophagy through multiple different mechanisms. We hypothesize that enhancing SIRT1 can induce autophagy to confer cytoprotection against liver I/R injury. SIRT1 was genetically manipulated using an adenovirus expressing SIRT1 (AdSIRT1) or Tamoxifen inducible SIRT1 knockout (SIRT1 KO) mice. Pharmacological activation of SIRT1 was performed using putative SIRT1 activators, Resveratrol (RSV) and SRT1720. SIRT1 overexpression and genetic ablation lead to autophagy induced cytoprotection and hypersensitization to I/R, respectively. Under nutrient rich conditions, SIRT1 induced autophagy was independent of mTOR inhibition and AMPK activation, but led to an increase the ATG7 expression, which may play a role in the enhanced basal autophagic flux. Electron, confocal, and intravital multiphoton microscopy were performed to confirm these finding by analyzing the mitochondrial ultrastructure, mitochondrial bioenergetics, the mitochondrial permeability transition (MPT), autophagosome generation and autophagic flux. These data all demonstrate that SIRT1 overexpression and activation enhances autophagy to suppress mitochondrial dysfunction and liver I/R injury.
Background

Mitochondrial dysfunction is the causative mechanism leading to hepatocyte death during warm I/R injury (45;57;58;311). Mitochondrial dysfunction occurs through the unregulated opening of the MPT pores (38;39;59). The MPT pore is a complex of several proteins located at contact site between inner and outer mitochondrial membranes (7) and reperfusion induced unregulated opening leads to the MPT onset, which is the lethal event. During reperfusion, the MPT onset is due to the influx of solutes up to 1500 Da into the mitochondrial matrix, which in turn leads to mitochondrial depolarization, impaired ATP production and ROS generation. As a result of osmotic homeostasis, the mitochondria begin to swell until the membrane ruptures and releases mitochondrial pro-apoptotic proteins into the cytosol leading to cell death (7;38;59;60). Mitochondrial dysfunction and ATP depletion in hepatocytes is the causative mechanism leading to cell death during I/R injury (45;57;58;311).

During reperfusion, mitochondrial Ca\(^{2+}\) overload leads to the MPT onset followed by generation of reactive oxygen species (ROS) to further permeabilize the mitochondrial membranes (39). Cyclosporine A, a cyclophilin D inhibitor, and mitochondrial Ca\(^{2+}\) chelation suppress the MPT onset and liver I/R injury (58;59). Clinically, these approaches are unfavorable due to the nephrotoxic effects of Cyclosporine A (312) and adverse effects from calcium imbalance in the vasculature (313). A novel strategy to circumvent the MPT onset is to eliminate signals triggering reperfusion injury by increasing mitochondrial turnover prior to I/R (47). Within a cell, the mitochondrial population is heterozygous and a small group of mitochondria initially undergoing the MPT onset promotes a sequential chain reaction to neighboring mitochondria causing the global MPT onset and depolarization (218). Clearance of this
discrete pool of the mitochondria may provide a novel approach to suppress liver I/R injury.

Autophagy is a lysosomal dependent catabolic process to remove long lived and dysfunctional organelles. Macroautophagy, referred to as autophagy, is a sequential process that involves several different autophagy related proteins (ATG) to initiation and develop autophagosomes, which are double membrane vacuoles that fuse with lysosomes. Autophagy occurs at a basal rate in cells to maintain homeostasis or can be stimulated by different pathological and physiological conditions. Autophagy impairment is a factor contributing to liver I/R injury, while the enhancement of autophagy is cytoprotective (51-53). Autophagy leads to the sequestration and degradation of damaged mitochondria after I/R (7;39;51-53). Post translational modifications including acetylation play a key role in mitochondrial biogenesis, bioenergetics and autophagy (208;260;269;314-317).

SIRT1 is a class III histone deacetylase that targets the transcription factors FOXO1 (190;202;274;318), FOXO3A (272;273;319) and PGC-1α (193;306;320) to regulate transcription of genes for mitochondrial biogenesis and mitochondrial autophagy. SIRT1 induced autophagy has been shown to suppress I/R injury in the heart (303;321;322), kidney (277), and brain (323;324), however SIRT1 inducing autophagy during liver I/R injury is unknown. Ischemic preconditioning and fasting are known approaches to induce cytoprotection against liver I/R injury and the protection is proposed to be mediated by SIRT1 (164;278). However, the role of SIRT1 conveying cytoprotection against liver I/R injury remains unclear.
The goal of this investigation was to determine whether modulating SIRT1 through genetic and pharmacologic approaches could affect the MPT onset and autophagy to suppress liver I/R injury. SIRT1 overexpression and activation induced autophagy, blocked the MPT onset and suppressed liver I/R injury. SIRT1 overexpression did not lead to the activation of AMPK or inhibition of mTOR, but increased ATG7 expression during normoxia. After I/R, SIRT1 overexpression did not cause a change the expression of several ATG proteins or the autophagy initiation signals but re-established autophagic flux. Collectively, this study suggests that SIRT1 induced autophagy provides cytoprotection against liver I/R injury.

Materials And Methods

Reagents And Drug Treatments

RSV was purchased from Sigma Aldrich (Cat number: R-5010) and SRT1720 was purchased from SelleckChem (Houston, TX). Cells were treated with RSV for 16 hours before hepatocytes underwent I/R, while SRT1720 treatment was for 1 hour before I/R. Both activators were continuously present during I/R.

In Vivo I/R And Adenoviral Injection

In vivo I/R injury was performed as described in Chapter 2.

Intravital Multiphoton Microscopy

To visualize autophagosomes and autophagic flux, livers were labeled with adenoviral GFP-LC3 or mCherry-GFP-LC3. After 20 minutes of reperfusion in vivo, a 24-gauge catheter was inserted into the portal vein. Rhodamine 123 (50 ml of 10 µM/animal), a ΔΨm-sensitive fluorophore, was infused for 10 minutes. The liver was gently withdrawn from the abdominal cavity and placed over a glass coverslip on the stage of a Zeiss LSM510 equipped with a multiphoton microscope. Images of green
fluorescing rhodamine 123 and GFP-LC3 were collected with a 40× water-immersion objective lens. Rhodamine 123 and GFP-LC3 were excited with 800 nm from a Chameleon Ultra Ti-Sapphire pulsed laser (Coherent Inc., Santa Clara, CA) and images were collected through 500-550-nm band pass filter. For imaging of mCherry-GFP-LC3, tandem fluorophores were excited at 800 nm and emission was separated through 500-530 nm (GFP) and 565-615 nm (mCherry) band pass filters. Ten images were randomly collected per each liver.

**Hepatocyte Adenoviral Infection**

Adenovirus expressing SIRT1 (AdSIRT1) was a kind gift from Dr. Junichi Sadoshima at The University of Medicine and Dentistry of New Jersey. Hepatocytes were infected with AdSIRT1 for 4 hours in hormonally defined medium (RPMI 1640 medium with no glutamine at pH 7.4, 0.3 mmol/L selenium, 1 µg/ml apo-transferrin, 100 nmol/L insulin, 1.5 µmol/L free fatty acids, 1% penicillin/streptomycin). After 4 hours, the medium was replaced with Waymouth medium and hepatocytes were incubated overnight (10-12 hours). For *in vivo* studies, mice were injected with AdLacZ, AdSIRT1, Ad-GFP-LC3 or Ad-mCherry-GFP-LC3 (7x10^{11} pfu/g) incubated for 2 days.

**Electron Microscopy**

A monolayer of hepatocytes were fixed using deoxygenated or oxygenated 2% paraformaldehyde and 2% glutaraldehyde, 125 mmol/L cacodylate, and 2.2 mmol/L CaCl at pH 6.2 for ischemia and pH 7.4 for reperfusion. Cells were fixed overnight (8-12 hours) then washed in a cacodylate buffer (100mmol/L cacodylate, 7% sucrose at pH 7.4) prior to staining with 1% osmium tetraoxide in the cacodylate buffer for 1 hour at room temperature. After staining, a Michaelis buffer (0.1 M HCl, 25% sucrose, 2.8 mmol/L sodium acetate trihydrate, 2.8 mmol/L sodium barbiturate) was used to wash
the cell prior to nuclear staining with a Kellenberger buffer (0.1 M HCl, 2.8 mmol/L sodium acetate trihydrate, 2.8 mmol/L sodium barbiturate, 2% uranyl acetate). Hepatocytes were then dehydrated using a 70%, 90% and 100% ethanol gradient for 15 minute intervals before en bloc. En bloc was performed using Embed-812, NADIC Methyl Anhydride, DMP-30 and DDSA by manufactures direction. All chemicals were purchased from Electron Microscopy Science (Hatfield, PA).

**Confocal Microscopy**

Confocal images were taken using an inverted Zeiss 510 laser scanning confocal microscope using tetramethylrhodamine methyl ester (TMRM), calcein-AM, and propidium iodide were collected with a gas-tight chamber (Zeiss, Jena, Germany)(39;51;297;297). Briefly, hepatocytes were seeded on glass bottom dishes overnight in Waymouth media. Hepatocytes were subjected to I/R as described previously in Chapter 2 with the addition of tetramethylrhodamine methyl ester (TMRM), Calcein-AM and propidium iodide 30 minutes prior to and continuously during reperfusion.

For autophagy analysis, hepatocytes were infected with AdLacZ or AdSIRT1 and Ad-GFP-LC3 or Ad-mCherry-GFP-LC3 seeded on glass bottom dishes (In Vitro Scientific, Sunnyvale CA) overnight in Waymouth media. Hepatocytes were subjected to I/R with the addition of TMRM 30 minutes prior to and continuously during reperfusion.

**Hepatocyte Isolation And Culture**

Hepatocytes were isolated as described in Chapter 2.

**Hepatocyte Simulated I/R**

Simulated I/R was performed as reported in Chapter 2.
Immunoblotting

Immunoblotting was performed as described in Chapter 2.

Tamoxifen Treatment For Inducible SIRT1 KO Mice

Tamoxifen treatment for SIRT1 KO was performed as described in Chapter 2.

Cell Death Assay Using Propidium Iodide

Hepatocyte death was determined as previously reported in Chapter 2.

Data Analysis

Statistics were performed as described in Chapter 2.

Results

SIRT1 Suppresses The MPT Onset And Hepatocyte Death.

To investigate if SIRT1 is cytoprotective, hepatocytes were infected with AdSIRT1 and subjected to I/R. Hepatocytes overexpressing SIRT1 suppressed hepatocyte death (Figure 3-1A) without influencing the endogenous reduction mechanism of SIRT1 (Figure 3-1B). The MPT onset causes hepatocyte death during warm I/R injury, thus we determined if SIRT1 overexpression prevented the opening of the MPT pore. We performed confocal microscopy to investigate the MPT onset and polarization status of the mitochondria using Calcein and TMRM (47;51;53) (Figure 3-1C). Indeed, SIRT1 overexpression resulted in sustained mitochondrial polarization after reperfusion and blocked the influx of calcein into the mitochondria, hence blocking the MPT onset. Electron microscopy was performed to evaluate the mitochondrial ultrastructure in hepatocytes overexpressing SIRT1. After 4 hours of ischemia, mitochondria remained circular morphology and maintain the integrity of inner and outer membranes during reperfusion in AdSIRT1 hepatocytes (Figure 3-1D). Collectively,
these data suggest that overexpression of SIRT1 suppresses the MPT onset, hepatocyte death and I/R injury.

To determine if pharmacological activation of SIRT1 could suppress I/R injury, we measured reperfusion induced cell death in hepatocytes treated with putative SIRT1 activators, RSV or SRT1720. Both RSV and SRT1720 suppressed hepatocyte death after I/R (Figure 3-2A), but without preventing SIRT1 depletion (Figure 3-2B). Electron microscopy was performed to evaluate the mitochondrial ultrastructure in hepatocytes treated with RSV. After 4 hours of ischemia, mitochondria maintained the normal morphology as well as the integrity of inner and outer membranes in hepatocytes treated with RSV, while control hepatocyte mitochondria were aberrant in shape and size (Figure 3-2C). Upon reperfusion, only the mitochondria from RSV treated hepatocytes retained mitochondrial membrane structures providing further evidence for the cytoprotective role of SIRT1 during liver I/R injury.

**SIRT1 Induces Autophagy To Suppress I/R Injury**

Autophagy clears unnecessary or dysfunctional proteins and organelles in a lysosome-dependent manner. Impaired autophagy contributes to liver I/R injury (51-53). To test if SIRT1-mediated cytoprotection is linked to autophagy, hepatocytes were subjected to I/R with and without SIRT1 overexpression and autophagic flux was assessed by analyzing LC3-II expression in the presence and absence of chloroquine (CQ), a lysosomal inhibitor (Figure 3-3A). Briefly, LC3-II is localized on the autophagosomal membrane and fusion with the lysosome leads to the degradation of LC3-II. Accordingly, the comparison of LC3-II before and after blocking lysosomal degradation using CQ can estimate autophagic flux. In the control hepatocytes, autophagic flux after I/R was marginal, consistent with previous data (51-53). However,
in the hepatocytes overexpressing SIRT1, the basal levels of LC3-II were significantly higher than the control cells and autophagic flux was also observed after I/R, suggesting that SIRT1 not only promotes basal autophagy but also prevents reperfusion-induced decline in autophagy. Increased autophagy by SIRT1 was confirmed in both fluorescence and electron microscopy. Imaging analysis of LC3 distribution in GFP-LC3 labelled hepatocytes showed that while control cells had few autophagosomes and diffused staining pattern, SIRT1 overexpressed cells encompassed numerous autophagosomes (green puncta) (Figure 3-3B). Dual staining of autophagosomes and mitochondria revealed that red fluorescing mitochondria were in close proximity to green fluorescing autophagosomes. Furthermore, electron micrographs showed that hepatocytes overexpressing SIRT1 had multiple autophagic vesicles (arrowheads) and, more importantly, structurally intact mitochondria after I/R (Figure 3-3C). Autophagic flux was also visualized in live cells with tandem mCherry-GFP-LC3 (53). GFP loses its fluorescence in the acidic environment of autolysosomes, whereas the red fluorescence of mCherry remains. As a consequence, autophagosomes and autolysosomes emerge as yellow and red puncta, respectively. Confocal imaging showed vast numbers of red puncta in SIRT1 overexpressed cells after 2 hours of reperfusion, implying a potent autolysosomal clearance by SIRT1 (Figure 3-3D). Altogether, these results highlight an integral role of SIRT1 in hepatocellular autophagy.

**SIRT1 KO Impaired Autophagy Sensitizes Hepatocytes To I/R Injury**

The importance of SIRT1 and its correlation to autophagy was further substantiated in SIRT1 KO hepatocytes. In WT hepatocytes, either RSV or SRT1720 significantly increased the basal autophagic flux (Figure 3-4A). However, neither activator was able to boost autophagy in SIRT1 KO cells. Similarly, SIRT1 KO
hepatocytes failed to sustain autophagic flux during a short-term I/R, a sub-lethal stress that does not induce death in wild type hepatocytes (Figure 3-4B). Of note, the addition of CQ to SIRT1 null cells under the normoxic condition failed to increase LC3-II levels, entailing that SIRT1 is necessary to retain the basal autophagic flux. To confirm that depletion of SIRT1 can sensitize hepatocytes to the MPT onset during reperfusion, we used the Tamoxifen inducible SIRT1 KO mice (298). Confocal imaging analysis further showed a rapid onset of the MPT in SIRT1 KO hepatocytes (Figure 3-4C), firmly indicating that the loss of SIRT1 sensitizes hepatocytes to the MPT onset and I/R injury. These results corroborate a pivotal role of SIRT1 in autophagy and ratify the necessity of SIRT1 in autophagy for hepatocyte survival during I/R.

**SIRT1 Induced Autophagy And Initiation Signals**

To explore the mechanism for SIRT1 induced autophagy, we investigated the mTOR and AMPK signaling pathway. Inhibition of mTOR and phosphorylation of AMPK are two canonical pathways to initiate autophagy (111;123). The mechanism of SIRT1 mediated induction of autophagy through these pathways has been controversial, thus we explored if SIRT1 overexpression can lead to mTOR inhibition or AMPK activation to initiate autophagy. SIRT1 overexpression did not decrease phosphorylation status of the downstream mTOR signaling protein p70s6k or increase in the phosphorylation status of AMPK under normoxic conditions (Figure 3-5A). Furthermore, the total levels of p70s6k and AMPK had significantly reduced during I/R, and SIRT1 overexpression did not change phosphorylation status of p70s6k or AMPK. Interestingly, AMPK is phosphorylated and p70s6k is dephosphorylated after 5 minutes of reperfusion. After 60 minutes of reperfusion, p70s6k becomes phosphorylated suggesting mTOR activation and retention of AMPK activation. These data suggest that during I/R hepatocytes are
initiating autophagy and the SIRT1 induced cytoprotection is a downstream event independent of mTOR and AMPK signaling cascade.

**SIRT1 Overexpression Increases ATG7 Expression**

Autophagy is a sequential process with multiple specific autophagy proteins involved in autophagy initiation, autophagosomal membrane elongation, autophagosome maturation, and lysosomal fusion for degradation of the constituents within an autophagosome. An increase in autophagy proteins is known to enhance autophagy (53), thus we investigated if SIRT1 induced autophagy leads to an increase in autophagic related proteins involved in these individual steps of autophagy. Overall, SIRT1 overexpression did not increase or decrease the expression levels of several proteins (Figure 3-5B). However, overexpression of SIRT1 did lead to a substantial increase in ATG7 expression during normoxia, which was lost during I/R. Previously, we have shown that overexpression of ATG7 enhances autophagy to suppress liver I/R injury. These studies suggest that SIRT1 induced autophagy may be mediated by increasing the expression of ATG7 under basal conditions.

**SIRT1 Overexpression Suppresses In Vivo I/R Injury**

To determine if SIRT1 induced could prevent liver I/R injury, we overexpressed SIRT1 in mice (Figure 3-6A). LC3-II immunoblot confirmed that a SIRT1 overexpression had affected autophagy before and after I/R by elevating the LC3-II expression (Figure 3-6B), which is consistent with our primary hepatocyte studies (Figure 3-3A). Intravital multiphoton images of Rhodamine 123 (Rd-123), a ΔΨm indicator showed an improved bioenergetics by SIRT1 (Figure 3-6C). While most Rd-123 fluorescence disappeared after reperfusion of the control livers, SIRT1 overexpressed livers displayed punctate, bright green fluorescence of Rd-123 in hepatocytes, indicative of polarized
mitochondria. Finally, to determine if SIRT1 can affect autophagy in vivo, we utilized the mCherry-GFP-LC3 adenovirus (Figure 3-6D). Upon reperfusion, liver tissue lost the mCherry-GFP-LC3 signal indicating a dysfunctional autophagy response, but overexpression of SIRT1 in livers led to the generation of autophagosomes (yellow) and autolysosomes (red) suggesting a functional autophagic response. This provides further evidence that SIRT1 conveys cytoprotection against liver I/R injury through autophagy.

Discussion

In these studies, we examined the role of SIRT1 during I/R injury in regards to mitochondrial dysfunction, the MPT onset and hepatocyte death. Here we demonstrate that SIRT1 overexpression and activation (a) suppressed the MPT onset and hepatocyte death; (b) increased autophagic flux before and after I/R; and (c) SIRT1 induced autophagy was independent of mTOR inhibition and AMPK activation but increased ATG7 expression under basal conditions. Our findings suggest that SIRT1 induced autophagy suppresses the MPT onset and liver I/R injury (Figure 3-7).

Hepatocyte death is initiated by mitochondrial dysfunction and the MPT onset during reperfusion (38;39;45). Reperfusion induced unregulated opening of the MPT pores is a lethal event. Consistent with previous studies (39;50;58), hepatocytes subjected to 4 hours of ischemia led to reperfusion induced death at 60 minutes, but overexpressing or activating SIRT1 suppressed reperfusion induced death (Figure 3-1 and Figure 3-2). In cardiomyoctes, SIRT1 increases mitochondrial antioxidants, Thioredoxin and Manganese superoxide, which may balance the ROS accumulation during reperfusion (259). However, hepatic mitochondrial ROS generation is a downstream event that proceeds the MPT onset in hepatocytes (39). Hepatocytes overexpressing SIRT1 were resistant to the mitochondrial dysfunction and the MPT
onset during reperfusion (Figure 3-1). Briefly, calcein labels both the nucleus and the cytosol when MPT pores are closed. However, the onset of the MPT redistributes calcein into the mitochondria while simultaneously releasing the TMRM, a mitochondrial membrane potential marker, into the cytosol. The mitochondria in the control cells underwent the MPT after I/R as indicated by the redistribution of calcein and TMRM, while mitochondria in SIRT1 overexpressing cells excluded calcein and retained TMRM in the polarized mitochondria. Mitochondrial dysfunction and the MPT onset leads to the rupture of the mitochondrial membranes to release the inner mitochondria contents (59). During reperfusion, electron micrographs clearly show ruptured mitochondria in control hepatocytes during reperfusion, but enhancing SIRT1 preserved intact mitochondrial membranes (Figure 3-1 and Figure 3-2). These results clearly show SIRT1 overexpression and activation suppresses liver I/R injury.

Autophagy allows for recycling and degradation of dysfunctional proteins and organelles through the lysosomal machinery, and conveys cytoprotection against liver I/R injury (51-53). Numerous studies support the essential role of SIRT1 in the induction of autophagy (164;189-192;260;317). Furthermore, SIRT1 induced autophagy removes damaged mitochondrial and attenuates oxidative damage during I/R (191;192;303). Consistent with these reports, we found overexpression or activation of SIRT1 induced autophagy by showing an accumulation of LC3-II in the presence and absence of chloroquine (CQ), a lysosome blocker (Figure 3-3). LC3-II is a key marker for autophagy because it localizes on the autophagosome, which are degraded by the lysosome. Application of CQ blocks lysosomal degradation and leads to an accumulation of LC3-II that can be measured by immunoblot. Autophagic flux was sustained in these viable
hepatocytes after I/R injury. Using a GFP-LC3 label we show that after I/R, hepatocytes are generating GFP labeled punctae (autophagosomes) in close proximity to mitochondria (Figure 3-3). The endoplasmic reticulum (ER) – mitochondrial contact sites are main sites for the autophagosome formation (325). These sites regulate the recruitment of autophagic machinery and donate membrane for the formation of the autophagosome (325). Using electron microscopy, we found that autophagosomes (double membrane vacuoles) are being generated in close proximity to the mitochondria and the ER, but could never identify mitochondria within an autophagosome. This may suggest that SIRT1 overexpression might play a role in Type III mitochondrial autophagy named micromitophagy (104). Autophagosomes sequester cargo, which is then transported to the lysosome for degradation. Autophagosomal-lysosomal fusion generates an autolysosome, which are single membrane vacuoles that contain several catabolic enzymes to degrade the autophagosomal cargo to be released back into the cytosol. After I/R, viable hepatocytes re-establish autophagic flux during reperfusion (Figure 3-3). Using a mCherry-GFP-LC3 label, we found that viable hepatocytes overexpressing SIRT1 generate autolysosomes after reperfusion, while control hepatocytes lack autolysosomes. These studies support that overexpression of SIRT1 prevents dysfunctional autophagy after I/R and promotes autophagic flux.

RSV and SRT170, agonists of SIRT1, enhance autophagy leading to cytoprotection against I/R injury (Figure 3-2), which coincides with previous findings (36;326-329). Interestingly, SIRT1 was depleted in these cells during I/R but cytoprotection against I/R injury was still conveyed. This may suggest that the activation of SIRT1 prior to I/R leads to changes in cellular mechanisms that convey the
cytoprotection against I/R injury, such as autophagy, even after I/R depletes SIRT1. SIRT1 mediating RSV induced autophagy has been controversial by recent evidence suggesting that RSV inhibits phosphodiesterases to increase cyclic AMP levels to simulate autophagy (250). Consistent with other reports (262;298), we found that RSV and SRT1720 induced autophagy is mediated by SIRT1 (Figure 3-2). Furthermore, we show that SIRT1 KO have an impaired autophagic flux and after 2 hours of ischemia followed by reperfusion autophagy remains dysfunctional (Figure 3-4) leading to hepatocytes death at a sublethal dose of I/R as shown in Figure 2-3. Overall, these data show that RSV and SRT1720 require SIRT1 to induce autophagy, and the loss of SIRT1 impairs agonist induced autophagy conveying cytoprotection against I/R injury.

Autophagy is a sequential process with an initiation followed by the recruitment of autophagic machinery for autophagosome generation. Mature autophagosomes then fuse with lysosomes to degrade autophagic cargo. Two well established autophagy initiation signals are AMPK phosphorylation and mTOR inhibition (111;123). Several studies report that RSV and SIRT1 overexpression increases AMPK phosphorylation (298;330). We found that SIRT1 overexpression does not enhance AMPK phosphorylation under normoxia or after I/R (Figure 3-5). This difference may be caused by the insulin supplementation in our in vitro hepatocyte model. Administration of insulin down regulates AMPK phosphorylation and increases lipogenic gene expression (331). While further studies are warranted to investigate this discrepancy, the use of this model provides mechanistic insights into SIRT1 induced autophagy independent of AMPK activation. Coinciding with previous studies (332;333), mTOR was not inhibited in primary hepatocytes by SIRT1 overexpression as indicated by an increase in p70s6k.
phosphorylation, a downstream target of mTOR (Figure 3-5). SIRT1 overexpression increases mTOR-p70\textsuperscript{S6K} interactions through deacetylation of the CTR region of p70\textsuperscript{S6K} to enhance mTOR phosphorylation of p70\textsuperscript{S6K} at thr\textsuperscript{389} (332). After I/R, overexpression of SIRT1 did not alter the phosphorylation status of AMPK or p70\textsuperscript{S6K}, which suggests that SIRT1 induced autophagy is independent of AMPK and mTOR autophagy initiation signals and a downstream target may be altered. As an important note, hepatocytes retain a strong autophagic initiation response through mTOR and AMPK upon reperfusion in hepatocytes independent of SIRT1. These studies suggest that overexpression of SIRT1 is enhancing basal autophagic flux without simulating mTOR or AMPK signaling cascades to activate autophagy.

Autophagy can be modulated by protein expression of lysosomal fusion and autophagy related proteins (53;190). Our evidence looking at several different autophagy and lysosomal proteins before and after I/R suggest that SIRT1 does not increase or decrease the lysosomal proteins (Lamp-2A, Cathepsin D) or several different autophagy related proteins (ATG3, ATG4B, ATG12-5 complex, ATG14L, Beclin 1, RUBICON) (Figure 3-5). However, SIRT1 overexpression did lead to an elevation in ATG7 expression under normoxic conditions and coincides with a previous finding (334), which was subsequently lost during I/R. ATG7 is an E1 ubiquitin like protein that facilitates LC3-II generation through ATP hydrolysis in both ubiquitin like pathways for LC3-II generation. Previously, we have shown that increasing ATG7 protein expression leads to autophagy activation and cytoprotection against liver I/R injury (52), thus suggesting that SIRT1 overexpression may be modulating basal autophagy flux through ATG7 up regulation. ATG5, ATG7 and ATG8 are all proteins involved in the elongation
of the autophagosomal membrane by generating the LC3-II, and are known substrates of SIRT1 for deacetylation (189). This may provide another avenue for SIRT1 to induce autophagy, however further studies are required to determine their involvement with enhanced autophagy.

Collectively, these in vitro studies demonstrate that SIRT1 overexpression enhances autophagy, blocks the MPT onset and suppresses I/R injury. To translate these findings into an in vivo model of liver I/R injury, we injected mice with the AdSIRT1 virus (Figure 3-6). Similar to the hepatocyte data, mice injected with SIRT1 led to (A) an increase in LC3-II expression before and after I/R injury implicating an altered autophagic response, (B) mitochondria contain a bioenergetic charge after reperfusion as indicated using Rhodamine 123, which accumulates in polarize mitochondria, and (C) a re-established autophagic flux after reperfusion as indicated by the formation of autophagosomes (yellow) and autolysosomes (red) using of the Ad-mCherry-GFP-LC3 vector. These data further support that SIRT1 induced autophagy suppresses in vivo liver I/R injury.

In summary, activation and overexpression of SIRT1 enhances basal autophagy in an mTOR inhibition and AMPK activation independent manner. During I/R, overexpression of SIRT1 prevents the MPT onset and promotes autophagy to suppress hepatocyte death. SIRT1 overexpression stimulates basal autophagy flux and increases ATG7 expression, which may play a role in the cytoprotection against I/R injury. However, SIRT1 overexpression did not affect autophagy initiation signals or autophagosome maturation proteins after I/R. This may suggest that SIRT1 is involved in another mechanism to sustain autophagic flux during reperfusion. Ultimately, this
evidence supports the role of SIRT1 induced autophagy as a cytoprotective mechanism against liver I/R injury.
Figure 3-1. SIRT1 overexpression suppresses I/R injury. (A) Hepatocytes overexpressing SIRT1 were analyzed by immunoblotting for SIRT1 expression. Hepatocytes infected with 10 MOI AdGFP or AdSIRT1 were subjected to 4 hours of ischemia followed by reperfusion to measure death using propidium iodide (n=5). (B) Representative immunoblot of SIRT1 expression in hepatocytes infected with AdSIRT1 during I/R. (C) Confocal images of hepatocytes infected with 10 MOI AdGFP or AdSIRT1 after 4 hours of ischemia and various reperfusion times in the presence of TMRM (Red-polarized mitochondria) and Calcein (Green). (D) Electron micrographs of hepatocytes infected with 10 MOI AdGFP or AdSIRT1 during I/R. **, p<0.01
Figure 3-2. Pharmacological activation of SIRT1 suppresses mitochondrial dysfunction and hepatocyte death. (A) Hepatocytes treated with 0.1 μM RSV for 16 hours or 0.1 μM SRT1720 for 1 hour prior to being subjected to 4 hours of ischemia followed by reperfusion to measure cell death. Activators were continuously present during I/R. (n=3) (B) SIRT1 was immunoblotted in hepatocytes treated with SIRT1 activators to analyze expression changes (n=3). (C) Representative micrographs of hepatocytes were treated with RSV and mitochondrial structure was analyzed before and after 4 hours of ischemia and 60 minutes of reperfusion.
Figure 3-3. SIRT1 overexpression induces autophagy to suppress I/R injury. (A) Hepatocytes infected with 10 MOI AdSIRT1 were subjected to I/R for LC3 immunoblot in the presence and absence of CQ. Hepatocytes were treated with 10 μM CQ for 1 hour prior to and continuously during I/R. (n=4) (B) Confocal images of hepatocytes infected with AdSIRT1 and GFP-LC3 after 4 hours of ischemia and 60 minutes of reperfusion. TMRM was used to indicate polarized mitochondria (Red). Autophagosomes (Green) (C) Electron micrographs of hepatocytes infected with AdGFP or AdSIRT1 before and after 4 hours of ischemia and 60 minutes of reperfusion. Arrow heads indicate autophagosomes. (D) Confocal images of hepatocytes infected with AdSIRT1 and AdmCherry-GFP-LC3 after 4 hours of ischemia and 2 hours of reperfusion. Autophagosomes (Yellow) and Autolysosomes (Red)
Figure 3-4. SIRT1 KO hepatocytes have an impaired autophagy leading to mitochondrial dysfunction and cell death. (A) WT and SIRT1 KO hepatocytes were treated with 0.1 μM RSV for 16 hours or 0.1 μM SRT1720 for 1 hour and autophagy flux was measured by LC3 immunoblot in the presence and absence of CQ. Hepatocytes were treated with 10 μM CQ for 1 hour. (n=3) (B) WT and SIRT1 KO hepatocytes were subjected to 2 hours of ischemia followed by 60 minutes of reperfusion in the presence and absence of CQ for autophagy flux analysis using LC3 immunoblot. Hepatocytes were treated with 10 μM CQ for 1 hour prior to and continuously during I/R. (C) Confocal images of hepatocytes treated with PI, TMRM and Calcein AM during reperfusion after 2 hours of ischemia. Arrows indicate PI stained nuclei and cell death.
Figure 3-5. Expression of autophagy proteins involved in initiation, elongation, and fusion in hepatocytes overexpressing SIRT1. Hepatocytes were infected with 10 MOI AdGFP or AdSIRT1 and subjected to I/R for analysis of autophagy proteins changes involved in (A) initiation (mTOR and AMPK) and (B) autophagosome maturation (ATG3, ATG4B, ATG7, ATG12-5, ATG14L, Beclin 1, RUBICON) and degradation (LAMP2A, Cathepsin D). (n=3)
Figure 3-6. Mice infected with AdSIRT1 prevent the MPT onset and induce autophagy during I/R. Mice were injection of either AdLacZ or SIRT1 at 7x10^{11}. Liver tissue was collected before and after 45 minutes of ischemia and 20 minutes of reperfusion for immunoblot analyzes of (A) SIRT1 or (B) LC3-II. (n=4) Representative images of mouse livers infected with AdLacZ or AdSIRT1 after 45 minutes of ischemia and 20 minutes of reperfusion (C) in the presence of Rhodamine123 to label polarized mitochondria or (D) livers infected with AdmCherry-GFP-LC3. Autolysosome (Red) and Autophagosome (Yellow).
Figure 3-7. Activation of SIRT1 suppresses I/R injury. Graphical interpretation for SIRT1 activation suppressing liver I/R injury. SIRT1 enhances autophagy under normoxic conditions, but does not suppress the endogenous ischemic reduction mechanism. Upon reperfusion, autophagy is initiated and the MPT onset is block to suppress hepatocyte death.
CHAPTER 4
SIRT1 CAN FORM A COMPLEX WITH MITOFUSINS AND SIRT1 INDUCED AUTOPHAGY IS MEDIATED THROUGH MFN2

Introduction

Chapters 3 described Sirtuin 1 (SIRT1) induced autophagy as a cytoprotection mechanism against liver ischemia/reperfusion (I/R) injury, however the mechanism of SIRT1 inducing autophagy during reperfusion remains unclear. In this chapter, we investigated potential targets of SIRT1 that may lead to the sustaining autophagy during reperfusion. Mitochondrial accumulation of SIRT1 has been proposed to mediate cytoprotection against I/R injury. Mitofusin 2 (MFN2) is an outer mitochondrial membrane protein that regulates autophagosomal-lysosomal fusion. We hypothesize that MFN2 plays a role in SIRT1 mitochondrial localization and enhanced autophagy.

Using a combination of immunoprecipitation and immunoblot from an enhanced hepatocyte mitochondrial fraction, we have identified a novel SIRT1-mitofusin complex under nutrient rich conditions. Moreover, overexpression of SIRT1 led to the deacetylation of MFN2. The importance of MFN2 in SIRT1 induced autophagy and I/R injury was confirmed using an adenovirus expressing short hairpin MFN2 (AdShMFN2). Genetic ablation of MFN2 led to attenuated autophagy and hypersensitivity to I/R, which SIRT1 overexpression could not prevent. This study shows that MFN2 mediates SIRT1 induced autophagy to convey cytoprotection against I/R injury.

Background

SIRT1 induced autophagy is a cytoprotective mechanism to suppress I/R in multiple different organs. Autophagy removes damaged and dysfunctional mitochondria to suppress tissue death during I/R (47;51;52). SIRT1 is a lysine deacetylase that removes an acetyl group on a lysine residue to expose a positively charge ε-amino
group (335;336). Deacetylation can change characteristics and functions of the target substrate in diverse ways that including protein-protein interactions, protein stability, and enzymatic activity (189;332;337-339). SIRT1 localizes to the nucleus and cytosol, nuclear SIRT1 deacetylates transcription factors to enhance protein expression involved in mitochondrial biogenesis and autophagy, while cytosolic SIRT1 interacts with autophagy proteins ATG7, LC3, and the ATG12-5 complex to enhance activity (189). However, the mechanism for SIRT1 induced autophagy during liver I/R remains unclear. Recently, it has been proposed that a small fraction of SIRT1 localizes to the mitochondria (196;276). Furthermore, ischemic preconditioning increases SIRT1 activity and localization to the mitochondria to protect against I/R injury (276). However, the mechanism for SIRT1 localizing to the mitochondria and suppressing I/R injury remains unclear.

Over one-third of all mitochondrial proteins are acetylated with the majority (53%) containing one or two acetylation sites (315). Mitochondrial protein deacetylation plays a crucial role in the clearance of mitochondria through autophagy (269). Mitofusins (MFNs) are large dynamin-related GTPases with two isoforms, MFN1 and MFN2. These mitofusins are over 80% homologous (340) and localize on the outer mitochondrial membrane (340-343). MFN1 is found exclusively on the mitochondria, while MFN2 has been found on mitochondria and endoplasmic reticulum (ER) at the ER-mitochondria contact sites (325;344). These contact sites have been implicated in play a critical role in the generation of autophagosomes (325).

MFN2 was originally identified as a mediator in mitochondrial fusion (345), however recent evidence implicates MFN2 can play a role in several other processes
including mitochondrial Ca\(^{2+}\) homoeostasis, fission/fusion, mitochondrial autophagy, oxygen consumption, and energy production (340;344;346-348). MFN2 is proposed to have a pivotal role in mitochondrial autophagy through two mechanisms. (A) The PTEN induced putative kinase 1 (PINK1)/Parkin system alters MFN2 by post translational modifications that lead to mitophagy (349). (B) MFN2 recruitments the ATG14L-Beclin-PI3KIII complex to the ER-mitochondria contact sites for autophagosomal formation mediated by the Stx17, a Q-SNARE, and facilities the insertion of Rab7, a fusion protein, onto the autophagosomal membrane (325;348), however the role of MFN2 in SIRT1 induced autophagy is unknown.

The goal of this investigation was to determine potential targets of SIRT1, the acetylation status of the substrate proteins, and their role in SIRT1 induced autophagy. Hepatocyte acetylation was determined using protein extracts and immunoblot with an Acetyl-lysine (Acetyl-K) antibody. SIRT1 immunoprecipitation followed by immunoblot was performed to determine SIRT1 interacts with MFN1 and MFN2. Acetylation of both MFN1 and MFN2 was confirmed in human liver tissue and hepatocytes by immunoprecipitation and immunoblot. Overexpression of SIRT1 leads to deacetylation of MFN2, but not MFN1. Next, we analyzed the role of MFN2 in SIRT1 induced autophagy and cytoprotection against I/R injury. MFN2 deficient hepatocytes impaired SIRT1 induced autophagy and lost cytoprotection against I/R injury. Furthermore, MFN2 deficiency alone leads to impaired autophagy and hypersensitivity to sublethal I/R conditions as compared to control. Collectively, this study shows that MFN2 mediates SIRT1 induced autophagy.
Materials And Methods

Human Liver Tissue Collection

Human liver tissue was collected as described in Chapter 2.

Human Tissue Cytosolic And Membrane Subfractionation

Human tissue was homogenized in KRH containing protease and phosphatase inhibitors (1:1000) followed by two 1 minute centrifugations at 600 x g. The supernatant was collected and centrifuged at twice at 21,000 x g at 4°C for 15 minutes for the C fraction (supernatant). The pellet was flash frozen in liquid nitrogen and thawed on ice. The pellet was suspended in 500 µl hypotonic solution (10 mM Tris pH 7.6) followed by an additional homogenization to ensure plasma membrane disruption. The homogenate was centrifuged at 600 x g at 4°C for 5 minutes to collect the supernatant, which was performed 3 times. The supernatant was collected and centrifuged at 14,000 x g at 4°C for 10 minutes. The pellet was suspended in RIPA or cell lysis buffer for immunoblot or immunoprecipitation followed by an on ice incubation for 15 minutes. To collect the M fraction, the samples were centrifuged at 21x000 x g at 4°C for 15 minutes and the supernatant was collect.

Hepatocyte Isolation And Culture

Hepatocytes were isolated as described in Chapter 2.

Hepatocyte Adenoviral Infection

Adenoviral infection was performed as described in Chapter 3.

Hepatocyte Cytosolic And Membrane Subfractionation

Primary hepatocytes (3.5 x 10⁶) were seeded in a 100 mm and incubated overnight in Waymouth media. Subfractionation was modified for isolated primary hepatocytes as described in fibroblast (350). Briefly, hepatocytes were scraped in KRH
containing protease and phosphatase inhibitors (1:1000) followed by 1 minute centrifugation at 1,000 x g. The supernatant was collected and centrifuged at 21,000 x g at 4° C for 15 minutes to collect the supernatant as the soluble fraction. The pellet was flash frozen in liquid nitrogen and thawed on ice. The pellet suspended in 500 µl hypotonic solution (10 mM Tris pH 7.6) was followed by homogenization to ensure plasma membrane disruption. The homogenate was centrifuged at 600 x g at 4° C for 5 minutes. The supernatant was collected and centrifuged at 14,000 x g at 4° C for 10 minutes. The pellet was suspended in RIPA or cell lysis buffer for immunoblot or immunoprecipitation followed by and ice incubation for 15 minutes, respectively. To collect the M fraction, the samples were centrifuged at 21,000 x g at 4° C for 15 minutes and the supernatant was collect.

**Immunoprecipitation**

For immunoprecipitation, the lysates from the M fractions were pooled together from 3 different mouse hepatocyte isolation using 60 x 10⁶ hepatocytes per mouse for a total protein concentration between 250-350 µg. Using the pooled M fraction, 250 µg of protein was incubated overnight with antibodies at 4° C overnight. For immunoprecipitation, Acetyl-K antibody was purchased from ImmuneChem (Burnaby, British Colombia), while FOXO1 and FOXO3A antibodies were purchased from Abcam (Cambridge, MA). All other antibodies were used to immunoprecipitation were the same antibodies used for immunoblot. Immunoprecipitates were incubated with Protein A/G Plus Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at 4° C then eluted in 2x Laemmli buffer at 95°C. Samples were separated on polyacrylamide gels then electrophoretically transferred to nitrocellulose membrane (Watman GmbH,
Dassel, Germany). Rabbit and mouse immunoglobulin G purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA) were used as control for nonspecific binding.

**Immunoblotting**

Immunoblotting was performed as described in Chapter 2. FOXO1, MFN2 and MFN2 antibodies were purchased from Abcam (Cambridge, MA). VDAC, Cox IV, Lamin B, FOXO3A, and Acetylated Lysine (Acetyl-K) were purchased from Cell Signaling (Danvers, MA). PGC-1α was purchased from Novus Biologicals (Littleton, CO).

**Tamoxifen Treatment For Inducible SIRT1 KO Mice**

Hepatocyte death was determined as previously reported in Chapter 2.

**Hepatocyte Adenoviral Infection**

Adenoviral infection for AdGFP and AdSIRT1 was performed as described in Chapter 3. For knockdown of MFN2, adenovirus harboring a small hairpin RNA targeting MFN2 (GCTACAGCTCATCATCAGTTA) was constructed (AdshMFN2). AdshSCR and AdshMFN2 infection was performed following AdGFP and AdSIRT1 infection. Hepatocytes were infected with AdshSCR or AdshMFN2 for 16 hours in hormonally defined medium (RPMI 1640 medium with no glutamine at pH 7.4, 0.3 mmol/L selenium, 1 µg/ml apo-transferrin, 100 nmol/L insulin, 1.5 µmol/L free fatty acids, 1% penicillin/streptomycin).

**Data Analysis**

Statistics were performed as described in Chapter 2.
Results

Novel Mitofusin-SIRT1 Complexes

SIRT1 is a class III histone deacetylase that targets multiple non-histone targets. Protein acetylation is a post translation modification that can regulate autophagy (189;190;269;351). To determine the changes in acetylation status, immunoblotting was performed with acetyl-K antibody. Whole cell protein extracts did not detect noticeable changes in acetylation status upon SIRT1 overexpression (Figure 4-1A). Next, subcellular fractionation suggested that SIRT1 accumulates in the membrane fraction, especially with higher titers of AdSIRT1 (Figure 4-1B). Immunoblotting with organelle-specific markers and acetyl-K antibody showed that the membrane fraction contained both the nucleus and the mitochondria (Figure 4-1C) and SIRT1 overexpression deacetylated some proteins (arrow heads) (Figure 4-1D). This suggests that proteins in the membrane fraction may be potential substrates for SIRT1 deacetylation. The localization and acetylation status of the known SIRT1 substrates FOXO1 (202;274;318), FOXO3A (273;319) and PGC-1α (193;320), were determined (Figure 4-2). FOXO1 may have been deacetylated, but the cytosolic localization would not lead to gene transcription. FOXO3A and PGC-1α were localized to the membrane fraction that contained the nucleus, but no detectable deacetylation event was observed. Thus, we focused on trying to identify potential new substrates for SIRT1 on the mitochondrial outer membrane. Using the M fraction and SIRT1 immunoprecipitation, we successfully identified MFN1 and MFN2 protein complex with SIRT1, but not with voltage dependent anion channel (VDAC) (Figure 4-3A). Next, we explored if SIRT1 can deacetylate either MFN1 or MFN2, or both. SIRT1 overexpression substantially reduced the levels of
acetylated MFN2, but not of MFN1 (Figure 4-3B). MFN2 appears to be endogenously acetylated in both human livers and SIRT1 KO hepatocytes (Figure 4-3C and Figure 4-3D). This suggests that MFN2 is a SIRT1 substrate and endogenously acetylated independent of SIRT1.

**MFN2 Mediates SIRT1 Induced Cytoprotection Against Liver I/R Injury**

MFN2 has recently been proposed to play a crucial role in autophagy (325;348). SIRT1 induced autophagy provides cytoprotection against I/R injury, thus we investigated if MFN2 is mediating SIRT1-induced cytoprotection. Hepatocytes infected with AdshMFN2 significantly decreased MFN2 expression under basal conditions to approximately 64% compared to the control (Figure 4-4A). Importantly, knockdown of MFN2 abrogated cytoprotection conferred by SIRT1 overexpression demonstrating the crucial role of MFN2 in SIRT1-dependent cytoprotection against I/R injury (Figure 4-4B). As SIRT1 overexpression induces autophagy to protect against liver I/R injury, autophagic flux was assessed in hepatocytes with deficient MFN2 expression after prolonged I/R. The autophagic flux sustained by SIRT1 overexpression after 2 hours of reperfusion disappeared after silencing MFN2 (Figure 4-4C). MFN2 deficient hepatocytes had LC3-II levels comparable to control cells. Moreover, we analyzed autophagic flux in normoxic cells in the presence and absence of SIRT1 overexpression (Figure 4-4D). Basal autophagic flux prior to I/R was also markedly reduced by MFN2 knockdown, suggesting that MFN2 is required for basal autophagy. In agreement with our findings, SIRT1 overexpression noticeably increased the levels of LC3-II both before and after the administration of CQ. However, silencing of MFN2 virtually abolished these increases, reinstating the importance of MFN2 in both basal and SIRT1-mediated autophagy.
To further investigate MFN2 knock down impaired autophagy, some hepatocytes were subjected to 2 hours of ischemia followed by reperfusion, which is a sublethal condition in control hepatocytes. Cell death after reperfusion was minimal in the hepatocytes treated with scrambled shRNA, but a significant increase in cell death was observed in MFN2 deficient cells (Figure 4-5A). Furthermore, knockdown of MFN2 did not affect the expression of SIRT1 and MFN1 during I/R (Figure 4-5B), but analysis of LC3-II demonstrated that silencing of MFN2 arrested autophagic flux (Figure 4-5C) and hepatocytes underwent reperfusion induced death. This evidence suggests that MFN2 mediates SIRT1 induced cytoprotection against liver I/R injury.

Discussion

In these studies, we investigated potential targets of SIRT1. Based on our findings, we propose that (a) SIRT1 forms a complex with MFN1 and MFN2, which are endogenously acetylated; (b) MFN2 is a substrate for SIRT1 deacetylation; (c) SIRT1 induced autophagy and cytoprotection against I/R injury is mediated by MFN2; and (d) the loss of MFN2 sensitizes hepatocytes to I/R injury. Overall, we conclude that MFN2 plays an important role in autophagy regulation and that overexpression of SIRT1 deacetylates MFN2, which may play a role in suppressing liver I/R injury (Figure 4-6).

Initially, we anticipated that overexpression of SIRT1 would deacetylate FOXO1, FOXO3A or PGC-1α, because several studies suggest this occurrence under nutrient starvation, high fat diets, and oxidative stress (193;202;273;274;318-320). However, we could not observe that overexpression of SIRT1 in primary hepatocytes deacetylates the endogenous levels of FOXO1, FOXO3A or PGC-1α under nutrient rich conditions (Figure 4-2). Furthermore, SIRT1 overexpression did not increase the protein level of
the transcription factors target genes, which include ATG4B (352), ATG3 (352), Beclin 1 (113) and ATG14L (353) as shown in Figure 3-5. Hence, this evidence suggests that SIRT1 overexpression did not alter these transcription factors under these conditions.

SIRT1 has an imperative role in mitochondrial metabolism and a small fraction of SIRT1 may localize with the mitochondria (196;276). It is unknown if SIRT1 contains a mitochondrial targeting sequence, which is required for transport into the mitochondrion. With recent studies suggesting SIRT1 localizes to the mitochondria, we set out to determine if SIRT1 could be interacting with the outer mitochondrial membrane proteins MFN2, MFN1, and VDAC (Figure 4-3). Interestingly, we found that SIRT1 can form a complex with both mitofusins. MFN1 and MFN2 are known to form homodimers and heterodimers for mitochondria fusion and stabilize the mitochondria to the ER membrane(344) implicating that SIRT1 may localize to the ER-mitochondrial contact sites to regulate autophagosome generation. Further investigations are required to determine the localization and possible linker proteins involved in this SIRT1-Mitofusin complex.

In a proteomics study, using MV4-11 cells, a human myeloid leukemia cell line, MFN2 was discovered to be acetylated (354). Using a combination of subfractionation, immunoprecipitation and immunoblot, we identified MFN2 and MFN1 as acetylated proteins (Figure 4-3). Overexpression of SIRT1 leads to deacetylation of MFN2, but not MFN1 suggesting that MFN2 is a substrate for SIRT1. Deacetylation of MFN1 represses ubiquitination and degradation (355), but functional roles of MFN2 deacetylation is largely unknown. Mouse MFN2 contains forty-one lysine residues with four residues (Lys^{37,215,355,654}) located within the SIRT1 consensus sequence X_{5}-K(Ac)-{Y,W,F}-X_{5} or
X6-K(Ac)-X5 - {Y,W,F} (245). Further studies are required to clarify the acetylation sites of MFN2. Furthermore, SIRT1 KO did not lead to an accumulation of acetylated MFN2 (Figure 4-3) suggesting that MFN2 acetylation is independent of SIRT1.

Impaired mitochondrial autophagy leads to the accumulation of dysfunctional mitochondria and cell death during I/R (39;45;51;52). Mitochondrial autophagy is a complex process with distinctive mechanisms. In neurons, mitochondrial depolarization leads to mitophagy through the PINK1/Parkin system, which involves ubiquitination and phosphorylation of MFN2 to stimulate autophagy (151;356). In reticulocytes, NIX is used as the linker protein to eliminate mitochondria through autophagy in a manner independent of the PINK1/Parkin (154) with no known role for MFN2. Recently, the mitochondrial acetyltransferase GCN5L has been proposed to play a role in mitochondrial autophagy (270). GCN5L null cells contain mitochondria that are globally deacetylated, which in turn led to the mitochondria accumulating LC3-II to facilitate mitophagy implying that acetylation status is an important regulatory mechanism for mitochondrial autophagy (270;271). SIRT1 induced autophagy is cytoprotective against liver I/R injury, but MFN2 deficient hepatocytes lose cytoprotection and autophagy flux (Figure 4-4). This suggests that MFN2 has an important role in SIRT1 induced autophagy and cytoprotection against I/R injury. Coinciding with previous findings (325;357), MFN2 deficiency caused an impaired basal autophagy. Furthermore, MFN2 mediated SIRT1 induced autophagy under basal conditions and after I/R (Figure 4-4). Myocytes and MEF cell lines show that the depletion of MFN2 decreases mitochondrial membrane potential, altered Ca^{2+} homeostasis, enhanced mitochondrial fission, and impaired mitochondrial energetics (340;345;347;358;359). This may suggest that an
impaired autophagic response could be leading to the accumulation of dysfunctional mitochondria. Impaired basal autophagy flux by MFN2 knock down also resulted in hypersensitivity to sublethal I/R conditions (Figure 4-5), further supporting that impaired autophagy is an imperative factor leading to I/R injury.

MFN2 depletion has been proposed to convey cytoprotective against I/R in cardiomyoctes by suppressing Ca\(^{2+}\) influx and the MPT onset, however MFN2 depletion only delays cell death, which occurs through an impairment in the fusion between autophagosomes and lysosomes at later stages (348). This delay may be attributed to an impaired mitochondrial repolarization in MFN2 depleted cells that already have a defect in establishing mitochondrial membrane potential prior to I/R. Mitochondrial repolarization upon reperfusion leads to the mitochondrial Ca\(^{2+}\) accumulation (45). The calcium uniporter facilitating the Ca\(^{2+}\) accumulation is dependent on mitochondrial membrane potential, which in turn leads to cell death (39). Further studies are required to investigate the re-established mitochondrial membrane potential upon reperfusion in MFN2 KO cells. MFN2 depleted cells causes several metabolic alterations including lower oxygen consumption rates, reducing substrate oxidation, decreasing mitochondrial oxidative phosphorylation, and increasing anaerobic glycolysis of glucose but lowering the rates for glycogen synthesis (345;360). In comparison, primary hepatocytes are highly dependent on aerobic respiration and utilize glycogen driven anaerobic glycolysis to prevent ATP depletion (5), and the inability to process glycogen efficiently may be another factor leading to the hepatocyte sensitization to I/R injury. Further investigations to clarify the role of MFN2 during liver I/R injury are required.
Overall, we conclude that MFN1 and MFN2 are endogenously acetylated in human livers and mouse hepatocytes. SIRT1 can form a complex with MFN1 and MFN2, but only deacetylate the latter. Furthermore, MFN2 plays a critical role in basal and SIRT1 induced autophagy to convey cytoprotection against hepatic I/R injury.
Figure 4-1. Acetylation changes in SIRT1 overexpressing hepatocytes. Hepatocytes were infected with 10 MOI AdGFP or AdSIRT1. (A) Representative immunoblot of lysine acetylation from whole hepatocyte protein lysates after adenoviral SIRT1 infection. (n=3) (B) Representative immunoblot of hepatocyte subfractionation and localization of SIRT1 at different AdSIRT1 MOIs. (C) Representative immunoblot for organelle identification in the cytosolic (C) and membrane (M) fractions after hepatocyte subfractionation using markers for the ER (calnexin), nucleus (lamin B), mitochondria (MFN1, MFN2, Cox IV, VDAC) and cytosol (α-tubulin). (D) Representative immunoblot of acetylated proteins in the cytosolic and membrane fractions of SIRT1 overexpressing hepatocytes.
Figure 4-2. PGC-1α, FOXO1 and FOXO3A localization and acetylation changes in isolated hepatocytes after SIRT1 overexpression. Hepatocytes were infected with 10 MOI AdGFP or AdSIRT1 followed by subfractionated into the C and M fractions. Using pooled M fractions from three separate hepatocyte isolations, we obtained 250 μg for immunoprecipitation using an Acetyl-lysine antibody, which was followed by immunoblot for PGC1-α, FOXO1 and FOXO3A.

Figure 4-3. MFN2 is a substrate of SIRT1. Hepatocytes were infected with 10 MOI AdGFP or AdSIRT1 and subfractionated into the C and M fractions. (A) Using pooled M fractions from three separate hepatocyte isolations, we obtained 250 μg for immunoprecipitation of SIRT1, which was followed by immunoblot for MFN2, MFN1, and VDAC. (B) Using the 250 μg of the M fraction, we immunoprecipitated acetylated proteins or MFN2 followed by immunoblot analysis. (C) Using the M fraction from wild type (WT) and SIRT1 KO mice, acetylated proteins were immunoprecipitated and analyzed for MFN2 using immunoblot. (D) Human liver tissue was subfractionated and using the M fraction we performed immunoprecipitation and immunoblot as indicated.
Figure 4-4. SIRT1 induced autophagy is impaired by MFN2 knockdown. (A) Hepatocytes were infected with different doses of AdShMFN2 to analyze protein expression using immunoblot. (n=3) (B) Hepatocytes infected with 20 MOI AdshSCR or AdshMFN2 and 10 MOI AdGFP or AdSIRT1 were subjected to 4 hours of ischemia followed by reperfusion to measure death. (n=3). (C) Autophagy flux was analyzed in hepatocytes infected with 20 MOI AdshSCR or AdshMFN2 and 10 MOI AdGFP or AdSIRT1 using LC3 immunoblot at 4 hours of ischemia and 120 minutes of reperfusion (n=3). Hepatocytes were treated with 10 μM CQ for 1 hour prior to and continuously during I/R. (D) Hepatocytes were infected with 20 MOI AdshSCR or AdshMFN2 and 10 MOI AdGFP or AdSIRT1 for LC3 immunoblot in the presence and absence of CQ. Hepatocytes were treated with 10 μM CQ for 1 hour. (n=3)
Figure 4-5. MFN2 deficient hepatocytes are sensitive to I/R injury. Hepatocytes were infected with 20 MOI AdshSCR or AdshMFN2 and subjected to 2 hours of ischemia followed by reperfusion to measure (A) cell death and (B) protein expression of SIRT1, MFN1 and MFN2 using immunoblot. (n=3) (C) Hepatocytes were infected with 20 MOI AdshSCR or AdshMFN2 and subjected to 2 hour of ischemia followed by reperfusion for autophagy flux analysis using LC3 immunoblot in the presence and absence of CQ. Hepatocytes were treated with 10 μM CQ for 1 hour prior to and continuously during I/R. (n=3)
Figure 4-6. MFN2 mediates SIRT1 induced autophagy to suppress the MPT onset and hepatocyte death during I/R. Graphical interpretation of the results in Chapters 4. SIRT1 overexpression leads to the deacetylation of MFN2 which may facilitate SIRT1 induced autophagy leading to cytoprotection against I/R injury. Studies using MFN2 knock down hepatocytes have an impaired autophagic flux and lose SIRT1 induced cytoprotection against I/R injury.
CHAPTER 5
SIRT1 OVEREXPRESSION SUPPRESSES THE LOSS OF MFN2 DURING LIVER ISCHEMIA/REPERFUSION INJURY

Introduction

Chapter 4 describes Mitofusin 2 (MFN2) mediating Sirtuin 1 (SIRT1) induced autophagy to convey cytoprotection against hepatic I/R injury. As shown in Chapter 3, autophagy initiation signals and autophagosome construction proteins were unaltered during reperfusion in SIRT1 overexpressing hepatocytes implicating an unidentified alteration was leading to sustained autophagic flux. Taken together, this would imply that MFN2 is critical for SIRT1 induced autophagy during reperfusion. In this chapter, we investigate the role of MFN2 in SIRT1 induced cytoprotection against liver I/R injury. We hypothesize that SIRT1 overexpression prevents the loss of MFN2 during I/R to sustain autophagic flux. Human liver tissue collected from the transection margin confirmed the reduction of MFN2 during inflow occlusion-induced ischemia. Using mouse liver I/R and primary hepatocyte simulated I/R models, we show that MFN2 is depleted in mouse liver tissue and primary hepatocytes after prolonged ischemia. Inhibitor studies were performed in primary hepatocytes and determined that cathepsins and calpains are involved in the depletion of MFN2. Hepatocytes overexpressing SIRT1 and SIRT1 null hepatocytes were subject to I/R to identify changes in MFN2 expression. Overexpression of SIRT1 prevented the loss of MFN2 during I/R, while SIRT1 knock out enhanced the reduction of MFN2 at shorter ischemic times. These data shows that MFN2 depletion is associated with reperfusion induced hepatocyte death and that SIRT1 overexpression prevents the loss of MFN2, which may be a critical factor for the re-establishing autophagic flux and protection against liver I/R injury.
Background

Mitochondrial dysfunction is the causative mechanism underlying warm I/R injury within the initial phase (<2hours) of reperfusion (38;39;52;59). Upon reperfusion, the mitochondria undergo Ca^{2+} overload leading to the unregulated opening of the mitochondrial permeability transition (MPT) pore, which in turn leads to the generation of reactive oxygen species (ROS) (39;45). The accumulation of dysfunctional mitochondria generating reactive oxygen species further damages hepatocytes leading to the intermediate and late phases of reperfusion injury (5;47). Autophagy is the only known mechanism to sequester damaged and dysfunctional mitochondria for degradation. Autophagy impairment is a factor leading to the accumulation of dysfunctional mitochondrial and hepatocyte death (52).

MFN2 is an outer mitochondrial membrane protein that plays a key role in bioenergetics, ER-mitochondria membrane tethering, and mitochondrial autophagy (340;344;347;348;358). MFN2 knock out studies provide evidence supporting a dual role for MFN2 during I/R by showing that the loss of MFN2 suppresses the initial mitochondrial Ca^{2+} influx that would be lethal (359), but inevitably these cells succumbed to death due to impaired autophagy (348). Under basal conditions, the loss of MFN2 increases the expression of glycolytic genes to up regulate anaerobic glycolysis, represses mitochondrial oxidative phosphorylation, and increases the resistance to mitochondrial Ca^{2+} overload (345-347;358;359), which may lead to the initial protection against early reperfusion injury in MFN2 KO cardiomyoctes. However, as reperfusion continues autophagy is required to remove aberrant proteins and damaged organelles to maintain homeostasis. The loss of MFN2 impairs autophagosomal-lysosomal fusion through inability to recruit RAB7, a membrane fusion
protein, onto the autophagosomal membrane leading to the accumulation of autophagosomes and cell death (348).

Liver I/R leads to the degradation of MFN2 in mouse livers (361), however the mechanism for this reduction is unknown. The ubiquitin-proteasomal system degrades MFN2 in a sequential mechanism that involves 1) phosphorylation, 2) ubiquitination and 3) membrane extraction by p97, an AAA+ ATPase (349;356) prior to proteosomal degradation. However, the involvement of the ubiquitin-proteasomal system during I/R remains controversial (309). Calpains are cysteine proteases that become activated during I/R and cleavage cytosolic proteins leading to hepatocyte death (51;52;69), while cathepsins have been suggested to translocate to the cytosol and cleavage proteins during I/R. However, the involvement of calpains and cathepsins in the degradation of MFN2 is unknown.

The goal of this investigation was to determine the changes in MFN2 expression during liver I/R injury, and identify whether SIRT1 overexpression alters MFN2 levels upon reperfusion. Human and mouse livers subjected to inflow occlusion induced ischemia led to the reduction of MFN2. Using the in vitro simulated I/R model, we show that prolonging ischemia led to the depletion of MFN2 and hepatocyte death. Furthermore, we investigated the reduction mechanism leading to the depletion of MFN2 in primary hepatocytes, which was shown to involve cysteine proteases. Finally, we modulated SIRT1 expression to determine whether SIRT1 could play a role in the reduction of MFN2 during liver I/R injury. Our data suggests that SIRT1 overexpression suppresses MFN2 depletion during I/R, which may be a critical factor for to re-establishing autophagic flux and protection against liver I/R injury.
Materials And Methods

Reagents And Drug Treatments

Reagents and drug treatments were performed as described in Chapter 2.

Human Tissue Collection

Human liver tissue was collected as described in Chapter 2.

Mouse Liver In Vivo I/R And Adenoviral Injection

Mouse livers were subjected to in vivo I/R as described in Chapter 3.

RT-PCR And Qualitative PCR

For RT-PCR, cDNA (2 μL) was used with 1 μL of 100 nM primers for MFN2-Forward (5’-CCTGCCTTTTCCTACCTTG-‘3’), MFN2- Reverse (5’-ACAGGAAACGATGTGGGTCT-‘3’), β-actin-Forward (5’-GTGGGCGCTCTAGGCACCAA-‘3’), and β-actin-Reverse (5’-CTCTTTGATGTCACGCAGATTTC-3’). PCR was carried out using New England BioLabs One Taq DNA Polymerase system (Ipswich, MA) using the Eppendorf MasterCycler 5333 Version 2 (Hamburg, Germany). cDNA was denatured for at 95°C for 5 minutes followed by 32 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute.

For qPCR, cDNA was diluted 1 to 10 volumes with DNA/RNA free water from Fisher Scientific (Fair Lawn, New Jersey). SsoAdvanced SYBR Green Supermix was used as described by BioRad on a CFX-96 Real Time System C1000 Touch Thermocycler (Hercules, CA). qPCR was performed using the primers listed above at 95°C for 5 minutes followed by 39 cycles of 95°C for 10 seconds, 55°C for 30 seconds
and 65°C for 30 seconds followed by 5 cycles of 65°C for 30 seconds with 0.5°C increasing increments.

**Hepatocyte Isolation And Culture**
Hepatocytes were isolated as described in Chapter 2.

**Hepatocytes Simulated I/R Model**
Simulated I/R was performed as described in Chapter 2.

**Adenoviral Infection**
Adenoviral infection was performed as described in Chapters 3 and 4.

**Tamoxifen Inducible SIRT1 KO Mice**
SIRT1 KO was performed as described in Chapter 2.

**Immunoblotting**
Immunoblotting was performed as described in Chapter 2.

**Data Analysis**
Statistics were performed as described in Chapter 2.

**Results**

**Ischemia Depletes MFN2, And Reperfusion Sustains The Loss**
To investigate the change in MFN2 during liver I/R, human liver tissue subjected to 15 minutes of inflow occlusion induced ischemia was analyzed for MFN2 expression using immunoblot. MFN2 significantly decreased compared to controls (Figure 5-1A). Using our *in vivo* and *in vitro* mouse models of I/R injury, we confirmed that the prolonged ischemia leads to the depletion of MFN2 and does not recover during reperfusion (Figure 5-1B and Figure 5-1C). The loss of MFN2 was correlated with hepatocytes death as shown in Figure 2-2, and reperfusion after 2 hours of ischemia did
not lead to a further reduction in MFN2 (Figure 5-4C) and hepatocytes remained viable. To determine if MFN2 depletion is due to chemical instability or normal protein turnover, we treated hepatocytes with cycloheximide, a protein synthesis inhibitor (Figure 5-2A). MFN2 was stable up to 24 hours in Waymouth media suggesting that MFN2 is actively being degraded during I/R. Interestingly, the MFN2 mRNA was also targeted for degradation during I/R (Figure 5-2B), which further substantiated that preservation of the MFN2 protein during I/R may play an important role to I/R injury.

To investigate the mechanisms involved in the reduction of MFN2 during ischemia, primary hepatocytes were treated with inhibitors for the proteasome (MG-132), cathepsins (E64d) and calpains (ALLM). Although several studies indicate that MFN2 is ubiquitinated and degraded by the proteasome, hepatocytes treated with MG-132 did not suppress the loss of MFN2 during ischemia (Figure 5-3A). To explore the role of cathepsins, we treated hepatocytes with E64d, a pan cysteine cathepsin inhibitor (Figure 5-3B). In the presence of E64d, the reduction of MFN2 was substantially suppressed at 2 hours of ischemia, but extension of the ischemic time to 4 hours led to MFN2 depletion. As shown in Figures 2-7 and 3-4, after 2 hours of ischemia hepatocytes do not have autophagic flux, and MFN2 is still reduced. This could result from the lack of ATP during ischemia, which suppresses highly energy-dependent autophagic process. Prolonging ischemia leads to the activation of calpains (51-53). Inhibition of calpains using Acetyl-Leucine-Leucine-Methionine did not suppress the ischemic depletion of MFN2 (Figure 5-3C), thus suggesting that during prolonged ischemia MFN2 is depleted by an unknown protease(s). Surprisingly, a combination of cathepsin and calpain during reperfusion after 4 hours of ischemia led to a gradual
increase in MFN2 expression suggesting cysteine proteases are remaining active and degrading MFN2 during reperfusion (Figure 5-3D). This increase may suggest that reperfusion leads to MFN2 translation but active cysteine proteases prevent an accumulation, which may impair autophagy. Further studies focusing on reperfusion and protein translation are required. It is important to note that inhibition of cathepsins and calpains lead to an increased MFN2 expression during reperfusion, but only calpain inhibition prevented the loss of SIRT1 and provided protection against hepatic I/R injury as shown in Figures 2-5 and 2-7. This may suggest that preservation of MFN2 alone during reperfusion may not suppress I/R injury, and other proteins cleaved by calpains are required.

**Overexpression Of SIRT1 Suppresses MFN2 Depletion**

Previously, we have shown that SIRT1 induces autophagy after I/R and MFN2 is required for a functional autophagic flux. This would imply that during reperfusion MFN2 is required to establish autophagic flux in hepatocytes overexpressing SIRT1. Prior to I/R, Hepatocytes overexpressing SIRT1 did not have an significant elevation in MFN2 compared to the control under nutrient rich conditions (Figure 5-4A). During ischemia, the majority of MFN2 reduced in the presence or absence of AdSIRT1, but a small fraction of MFN2 was resistant to prolonged ischemia and reperfusion in SIRT1 overexpressing hepatocytes (Figure 5-4B). Furthermore, these cells did not accumulate MFN2 after 60 minutes of reperfusion suggesting that the active cysteine proteases may not target this MFN2 as a substrate. As shown in Figure 2-3, SIRT1 KO led to heightened sensitivity to sublethal conditions of I/R, thus we evaluated changes in MFN2 protein expression (Figure 5-4C). Surprisingly, SIRT1 KO led to an increase in MFN2 expression under basal conditions, but a greater reduction in MFN2 occurred
after 2 hours of ischemia, which did not recover during reperfusion. This may suggest that MFN2 in SIRT1 KO cells are more sensitive to proteolysis. Further investigations are required to identify potential proteases and activity leading to this reduction and the mechanism causing elevations in MFN2 under basal conditions. Collectively, these data suggest that SIRT1 plays a role in the degradation of MFN2 during hepatic I/R injury.

Autophagosome-lysosome fusion is mediated by MFN2 through the recruitment of Rab7 to the autophagosomal membrane (348). Hepatocytes overexpressing SIRT1 repress MFN2 depletion during I/R and have functional autophagosome-lysosome fusion indicated by the re-established autophagic flux during reperfusion. This suggests that inhibition of MFN2 loss by SIRT1 may lead to autophagy flux during reperfusion. SIRT1 overexpressing hepatocytes sustained autophagy flux at 120 minutes of reperfusion, which is impaired in MFN2 deficient hepatocytes subjected to similar conditions as shown in Figure 4-4. Under these conditions, MFN2 was sustained in hepatocytes infected with AdshSCR, but not in MFN2 deficient hepatocytes (Figure 5-4D), which further supports the notion that SIRT1 repressed MFN2 depletion leading to autophagosomal-lysosomal fusion during reperfusion for the re-establishment of autophagic flux. To determine if SIRT1 overexpression could suppress MFN2 depletion in the liver, we infected mice with AdSIRT1 and subjected them to \textit{in vivo} liver I/R injury (Figure 5-4E). MFN2 expression did not increase in liver prior to I/R, but MFN2 levels were significantly elevated upon reperfusion in livers overexpressing SIRT1, which was similar to our hepatocyte model. Overall, these studies show that SIRT1 overexpression suppresses the loss of MFN2 during I/R and supports our hypothesis that MFN2 mediates SIRT1 induced autophagy to suppress liver I/R injury.
Discussion

In these studies, we investigated MFN2 expression during liver I/R injury. Based on our findings, we propose that (a) both MFN2 mRNA and protein levels decrease during ischemia and remain depleted during reperfusion through cysteine proteases; (b) SIRT1 overexpression suppresses the loss of MFN2 and (c) MFN2 mediates SIRT1 induced autophagy during I/R injury. Overall, we conclude that multiple factors lead to the MFN2 depletion during I/R and SIRT1 overexpression prevents MFN2 depletion, which may play a role in re-establishing autophagic flux during reperfusion (Figure 5-5).

Mitochondrial dysfunction plays a central role leading to liver I/R injury. Studies have proposed that MFN2 has a critical role in autophagy (325;348) and Ca\textsuperscript{2+} regulation (344;347;359), which are two factors leading to hepatocyte death during I/R (39;45;47;51). An influx of Ca\textsuperscript{2+} leads to the unregulated opening of the MPT pore causing mitochondrial dysfunction (39). Impaired autophagy results in the accumulation of dysfunctional mitochondria releasing proapoptotic factors and generating ROS leading to cell death (47). MFN2 depletion studies show that dissociating the mitochondria from the ER can reduced Ca\textsuperscript{2+} influx in cardiomyocytes (344), but also alters bioenergetics by placing a higher demand on the glycolytic pathway for ATP production by increasing glycolytic gene expression and depressing mitochondrial oxidative phosphorylation (340;345;358). MFN2 depleted myocytes block the MPT onset but only delay death, which is caused by impaired autophagosome-lysosome fusion (348). MFN2 has been shown to be involved with liver I/R injury, however the role of MFN2 remains unknown.

Mouse livers subjected to liver I/R injury led to a reduction in MFN2, which coincides with a previous report (361) and resembles human livers that underwent 15
minutes of inflow occlusion induced ischemia (Figure 5-1). During warm I/R injury, damage to the parenchymal cells is the major factor leading to liver dysfunction and patient mortality (27;35;46). Using isolated hepatocytes, we confirmed that prolonged ischemia leads to the depletion of MFN2 that does not recover during reperfusion (Figure 5-1) and hepatocytes underwent reperfusion induced death as shown in Figure 2-2. To further clarify the role of ischemia in the depletion MFN2, we analyzed the protein stability of MFN2 and mRNA levels during I/R (Figure 5-2). Hepatocytes treated with cycloheximide, a protein synthesis inhibitor, maintained MFN2 expression up to 24 hours suggesting that MFN2 depletion is likely caused by ischemia. Furthermore, hepatocytes undergoing I/R have a significant reduction in MFN2 mRNA. This indicates that both the protein and mRNA are decreasing during ischemia leading to reperfusion induced hepatocyte death. Prolonged ischemia and hepatocyte death are causative factors leading to post liver resection failure, however the role of MFN2 during liver I/R in unclear (32-35).

Overexpression of MFN2 in hepatocytes suppressed mitochondrial dysfunction and cell death after I/R (361). This may suggest that preventing MFN2 depletion may also protected against I/R injury, however the MFN2 reduction mechanism is unknown, thus initially we set forth to characterize the proteases involved in the depletion of MFN2. MFN2 degradation occurs through post translational modifications that recruit AAA⁺ ATPase for MFN2 membrane extraction and transport to the proteasome for degradation (151;356). Proteasomal degradation is controversial during ischemia (309), thus we treated hepatocytes with inhibitors for the proteasome, cathepsins and calpains. Cathepsins are a family of lysosomal proteins the hydrolyze proteins but are
proposed to translocate to the cytosol, and remain active (71;73;76;83;362). Calpains are Ca\(^{2+}\) dependent cysteine proteases that increase in activity during prolonged ischemia (51;66;68;69;72). Proteasomal inhibition did not inhibit the loss of MFN2. Furthermore, we did not observe any motility shifts in MFN2 that would indicate ubiquitination (149;151;356). It is unknown whether cathepsins proteolyzes MFN2, however cathepsin inhibition does reduce cellular damage during I/R (67;75;83). Indeed, cysteine cathepsin inhibition suppressed the reduction of MFN2 at 2 hours of ischemia (Figure 5-3). Furthermore, prolonging ischemia led to the depletion of MFN2 in a manner independent of cathepsins and hepatocytes underwent reperfusion induced death as in Figure 2-7. Prolonging ischemia leads to heightened activity of calpains from the accumulation of intracellular Ca\(^{2+}\) levels (51;52). Inhibition of calpains did not suppress the loss of MFN2 during ischemia, but hepatocytes remained viable during reperfusion as shown in Figure 2-5. Thus, if MFN2 is required for autophagy induced cytoprotection, the levels should increase during reperfusion. Using the combination approach, we confirmed the re-establishment of MFN2 in the presence of cysteine protease inhibitors, E64d and ALLM. Based on these studies, we propose that MFN2 is degraded in two phases: (A) ischemic degradation occurs through the cysteine family of cathepsins and an unknown protease(s) lead to depletion and (B) activated cathepsins and calpains during reperfusion prevent the accumulation of MFN2 that may lead to the impairment in autophagy-lysosome fusion during reperfusion.

**Autophagy is a cytoprotective mechanism to suppress liver I/R injury (47).** As shown in previous chapters, SIRT1 induced autophagy is mediated by MFN2 to suppress liver I/R injury. This would imply that MFN2 must be expressed during liver I/R
in order for functional autophagy to occur and protect against liver I/R injury. SIRT1 deacetylates PGC1-α increases MFN2 expression (363). However, hepatocytes overexpressing SIRT1 did not have heightened MFN2 protein level, which further confirmed that PGC1-α is not deacetylated under nutrient rich conditions as shown in Chapter 4. The vast majority of MFN2 was depleted during ischemia, but a small fraction of MFN2 was resistant to degradation after 4 hours of ischemia. Hepatocytes overexpressing SIRT1 had approximately a 40% increase in MFN2 expression as compared to the control. Upon reperfusion, the sustained activation of cysteine proteases rapidly impaired the accumulation of MFN2, however the levels of MFN2 did not fluctuate in SIRT1 overexpressing hepatocytes. These data in combination with SIRT1 induced deacetylation of MFN2 as shown in Figure 4-3 may suggest that a small fraction of MFN2 is deacetylated to repress I/R induced proteolysis, which in turn would allow for a functional autophagosomal-lysosomal fusion and autophagy flux. This notion is supported by hepatocytes overexpressing SIRT1 sustain autophagic flux and repress MFN2 depletion after 4 hours of ischemia and 120 minutes reperfusion, which was impaired in MFN2 deficient hepatocytes. It would not be unusual for deacetylation to repress degradation, based on the deacetylation of MFN1 repressing ubiquitination and degradation (355). Further evidence to support that SIRT1 has a role in MFN2 proteolysis leading to dysfunctional autophagy and cell death was demonstrated in SIRT1 null hepatocytes. In sublethal I/R conditions, SIRT1 null hepatocytes after 2 hours of ischemia had a significant reduction in MFN2 that did not recover and autophagy flux was impaired leading to reperfusion induced cell death. More studies are required to investigate whether SIRT1 targets other substrates during short ischemic
times followed reperfusion. To translate these studies, we overexpressed SIRT1 in mice and confirmed that MFN2 expression was greater after liver I/R injury thus suggesting *in vivo* MFN2 depletion can be repressed by SIRT1 overexpression and that the repression of MFN2 may lead to functional autophagic flux during reperfusion.

Overall, these studies provide evidence for a possible mechanism involved in SIRT1 induced autophagy providing a functional autophagic response during reperfusion. These data suggests that SIRT1 deacetylation of MFN2 may repress the degradation and depletion of MFN2 during ischemia without impairing the endogenous degradation mechanisms, which in turn may lead to the functional autophagosome-lysosomal fusion and the degradation of dysfunctional mitochondria to provide cytoprotection against liver I/R injury.
Figure 5-1. MFN2 expression changes in human and mouse liver tissue and primary mouse hepatocytes during I/R. Immunoblot of MFN2 expression in (A) human liver tissue (n=3), (B) mouse liver tissue (n=3) and (C) mouse primary hepatocytes (n=4) subjected to indicated times of I/R. **,p<0.01 and ***,p<0.001.

Figure 5-2. MFN2 protein stability and mRNA expression during I/R in primary hepatocytes. (A) Immunoblot of MFN2 expression in hepatocytes treated with 35 μM cycloheximide (CHX) for various times. (n=3) (B) mRNA analysis using RT PCR and qPCR of MFN2 from primary hepatocytes during various times of I/R. (n=3). *** p<0.001
Figure 5-3. Inhibition of cysteine cathepsins suppresses the ischemic reduction of MFN2 in primary hepatocytes during I/R. (A) Immunoblot analysis of MFN2 expression during ischemia in cells treated with various concentrations of MG-132, a proteasome inhibitor. Hepatocytes were treated with MG-132 for 1 hour prior to and continuously during ischemia. (n=4) (B) Immunoblot analysis of MFN2 expression during ischemia in cells treated with various concentrations of E64d, a pan cysteine cathepsin protease inhibitor. Hepatocytes were treated with E64d for 1 hour prior to and continuously during ischemia. (n=4) (C) Immunoblot analysis of MFN2 expression during ischemia in cells treated with various concentrations of ALLM, a calpain inhibitor. Hepatocytes were treated with ALLM for 16 hour prior to and continuously during ischemia. (n=3) (D) Immunoblot analysis of MFN2 expression during ischemia in cells treated with 10 μM ALLM and 10 μM E64d. Hepatocytes were treated with ALLM for 16 hour and E64d for 1 hour prior to and continuously during ischemia. (n=3) *,p<0.05 and **,p<0.01
Figure 5-4. SIRT1 modulation affects MFN2 degradation during I/R. (A) Hepatocytes infected with 10 MOI AdGFP or AdSIRT1 were subjected to ischemia for MFN2 analysis using 15 μg of protein extract. (n=3) (B) Hepatocytes infected with 10 MOI AdGFP or AdSIRT1 were subjected to 4 hours of ischemia and 60 minutes of reperfusion for MFN2 analysis using 30 μg of protein extract. (n=3) (C) Wild type (WT) and SIRT1 KO hepatocytes were subjected to 2 hours of ischemia and reperfusion to analyze MFN2 expression using immunoblot. (n=3) (D) Hepatocytes infected with 10 MOI AdGFP or AdSIRT1 and 20 MOI AdshSCR or AdshMFN2 were subjected to 4 hours of ischemia and 120 minutes of reperfusion for MFN2 analysis using 30 μg of protein extract. (n=2) (E) Mice were injected with AdSIRT1 or AdLacZ followed by liver ischemia for 45 minutes and 20 minutes of reperfusion for MFN2 immunoblot. (n=4) *,p<0.05 and ** p<0.01
Figure 5-5. Overexpression of SIRT1 suppresses the MFN2 depletion during liver I/R injury. Graphical interpretation of SIRT1 overexpression leading to the preservation of MFN2 protein levels to suppress liver I/R injury.
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The research presented here explored the therapeutic potential for targeting SIRT1 to suppress liver I/R injury. At the time these studies began there were no investigations into the role of SIRT1 during liver I/R injury. Since that time two studies have been published implicating the importance of SIRT1 for cytoprotection against liver I/R injury in mice that were fasted or rats that underwent ischemic preconditioning. This research evaluated SIRT1 induced autophagy to suppress liver I/R injury. We are the first to report (A) a protein complex containing SIRT1 and mitofusins, (B) an acetylated MFN2 as a substrate for SIRT1, (C) MFN2 mediating SIRT1 induced autophagy, (D) calpain proteolysis of SIRT1 during I/R, (E) the biphasic proteolysis of MFN2 involving cysteine proteases during I/R, and (F) overexpression of SIRT1 leading to the repression of MFN2 proteolysis during I/R (Figure 6-1). Collectively, these studies support the therapeutic potential for targeting SIRT1 during liver I/R injury.

Autophagy is an endogenous cytoprotective mechanism against liver I/R injury. Autophagy stimulation may lead to remodeling of the heterogeneous mitochondrial population found within a cell to create a more resistant phenotype against I/R. Furthermore, autophagy can also support the removal of damaged mitochondria after I/R. SIRT1 and MFN2 both have been proposed to play a role in autophagy and I/R injury, however there is no known common mechanistic link between SIRT1 and MFN2. Human livers subjected to 15 minutes of ischemia led to the reduction of both SIRT1 and MFN2. Mouse livers and isolated hepatocytes exposed to prolonged ischemia led to the depletion of SIRT1 and MFN2, which provided the experimental models to
explore the mechanisms involved in the protein reduction, autophagy, and potential interactions.

This study provides evidence that SIRT1 and MFN2 have an integral role in I/R injury. Genetic ablation of either MFN2 or SIRT1 leads to heightened sensitivity to a sublethal dose of I/R. Both MFN2 knock down and SIRT1 knock out hepatocytes had a substantial amount of cell death during reperfusion after 2 hours of ischemia in comparison to control hepatocytes. Furthermore, SIRT1 and MFN2 protein levels are significantly reduced after 2 hours of ischemia but were sustained during reperfusion in hepatocytes, and these cells can remain viable. Prolonging the ischemic time to 4 hours caused SIRT1 and MFN2 depletion, and reperfusion induced mitochondrial dysfunction lead to cell death. Adenoviral overexpression of SIRT1 was used in hepatocytes and mouse livers to show that SIRT1 overexpression repressed MFN2 proteolysis, blocked the MPT onset, re-established autophagic flux and suppressed I/R injury. These cytoprotective effects of SIRT1 overexpression were subsequently lost in MFN2 deficient hepatocytes suggesting that MFN2 mediates SIRT1 induced cytoprotection against I/R injury (Figure 6-1).

The mechanisms involved in the proteolysis of SIRT1 and MFN2 further supported the important of maintaining these proteins during I/R. The reduction mechanisms involved in the depletion of SIRT1 and MFN2 during liver I/R was an unexplored area, thus we set forth to provide some mechanistic insight (Figure 6-1). To investigate potential mechanisms, hepatocytes were treated with inhibitors for the proteasome, cathepsins and calpains followed by simulated I/R to evaluate the protein levels. For clarify, the degradation can be broken down into 3 different stages: 2 hours
of ischemia (sublethal ischemia), 4 hours of ischemia (prolonged ischemia), and 60 minutes of reperfusion (reperfusion). The proteasome inhibitor, MG-132, failed to suppress the reduction of either SIRT1 or MFN2 at all stages. Sublethal ischemia leads to cathepsin proteolysis of SIRT1 and MFN2, while calpain inhibition had no effect. This suggests that cathepsin activation and degradation of SIRT1 and MFN2 does not lead to mitochondrial dysfunction and hepatocyte death. Prolonged ischemia leads to the depletion of SIRT1 and MFN2. Calpain inhibition only partially preserved SIRT1 without affecting MFN2 levels. Cathepsin inhibition had no affection on the depletion of SIRT1 and MFN2. This suggests that other proteases are being activated that leads to the reduction of SIRT1 and depletion of MFN2 between 2-4 hours of ischemia. Reperfusion sustained the depletion of SIRT1 and MFN2. Calpain inhibition maintained the prolonged ischemia SIRT1 level, while increasing MFN2 expression. Cathepsin inhibition did not alter the depletion of SIRT1, while increasing MFN2 expression. This suggests that upon reperfusion MFN2 may be translated but cysteine proteases prevent MFN2 accumulation leading to cell death. Moreover, cathepsin inhibition only suppress MFN2 leading to cell death, while calpain inhibition suppressed the depletion of both SIRT1 and MFN2 in addition to suppressing cell death, which clearly supports the importance of calpain activation leading to reperfusion induced cell death.

To establish a link between SIRT1 and MFN2, a series of experiments were performed on isolated hepatocytes and human liver tissue to achieve an enriched mitochondrial fraction for immunoprecipitation and immunoblotting. This study shows that SIRT1 can immunoprecipitate MFN2 and MFN1 suggesting a novel protein complex that is on the outer mitochondrial membrane or ER-mitochondria contact sites.
Furthermore, SIRT1 overexpression led to a decrease in the acetylated levels of MFN2 suggesting MFN2 is a substrate for SIRT1. MFN2 was endogenously acetylated in human liver tissue and hepatocytes from both wild type and SIRT1 KO mice suggesting that SIRT1 may not be involved in the acetylation process of MFN2. Further studies are warranted to identify the acetylation site(s) of MFN2, the protein interactions, and complex location.

Finally, this study shows that SIRT1 and MFN2 play a role in autophagy and MFN2 mediates SIRT1 induced autophagy to provide cytoprotection against I/R injury. SIRT1 knock out and MFN2 knock down impaired the autophagic response under basal conditions. Furthermore, SIRT1 induced autophagy was impaired by MFN2 knock down under similar condition, thus suggesting that MFN2 mediates SIRT1 induced autophagy and supports the crucial role of MFN2 in autophagy. Sublethal I/R in SIRT1 knock out and MFN2 knock down hepatocytes lead to dysfunctional autophagy during reperfusion resulting in hepatocyte death. Under nutrient rich conditions, SIRT1 overexpression led to an increase in ATG7 levels without alters autophagy initiation signaling suggesting a possible mechanism for SIRT1 induced autophagy that is dependent on MFN2. However, after I/R, SIRT1 overexpression did not change the autophagy initiation signals or the expression of proteins involved in autophagosome construction. During reperfusion, SIRT1 overexpression did not the inhibition of mTOR and activation of AMPK, thus the impairment may be downstream at the depletion of MFN2 leading to impaired autophagosome-lysosome fusion. SIRT1 overexpression repressed the depletion of MFN2 and enabled the re-establishment of autophagic flux during reperfusion. Further investigations are required to evaluate the role of MFN2 in the
autophagy mechanism before and after I/R injury. Overall, these studies suggest (1) an important role of MFN2 in the autophagy mechanism and (2) MFN2 mediates SIRT1 induced autophagy to protect against liver I/R injury.

**Clinical Application Of SIRT1 Activation**

Current approaches to suppress liver I/R injury are only beneficial for short ischemic times, cause vascular damage and prolong the surgical time. Pharmacological agents to suppress liver I/R injury target specific areas of a multifactorial mechanism and remain disappointing. A novel approach to suppress liver I/R injury is through the up regulation of autophagy (47), which is an endogenous mechanism to remove dysfunctional organelles and proteins. Impaired autophagy is a factor that contributes to liver I/R injury and enhancing autophagy conveys cytoprotection (47;51;53). SIRT1 is a pleiotropic protein that induces autophagy and protects against liver I/R injury by enhancing autophagy and repressing the loss of MFN2, thus targeting SIRT1 therapeutically may provide some clinical benefits. SIRT1 activators are being tested both experimentally and clinically for several different diseases. While the studies presented here focused mostly on overexpression of SIRT1, we have shown that RSV and SRT1720 can suppress hepatocyte death and enhance autophagy. Further studies are required to test the safety and applicability of using these SIRT1 agonists as therapeutics to suppress I/R injury. Studies have shown that RSV is cytoprotective against liver I/R injury in mice, but have been no clinical studies addressing RSV induced cytoprotection against liver I/R injury (36;326). Currently, phase 1 clinical trials have been performed testing the effects of SIRT1 agonists in several different diseases (Table 6-1), but no clinical study has investigated SIRT1 activation during liver I/R injury.
SIRT1 activation using SRT2104 in a clinical trial has a 2-4 hour $T_{\text{max}}$ and 16-18 hour half-life and appears to be safe with only minor side effects (364). However, some limitations for SIRT1 activation still need to be addressed. First, SIRT1 activation may provide cytoprotection against liver I/R injury, but is suggested to repress liver regeneration after mouse liver resection surgeries (198). Dose and time study optimizations for the administration of SIRT1 agonists need to be performed to address this potential limitation. Second, patients undergoing liver resection mostly have an underlying disease that may alter SIRT1 expression and activity to change cellular metabolism, and mitochondrial function. This may cause an adverse effect from SIRT1 activation during or after resection surgery, which can be addressed by using mouse liver disease models. Our study focuses on healthy livers undergoing prolonged ischemia and does not address the underlying liver diseases. Third, pharmacological activators can always provide a degree of nonspecific activation, which may cause adverse effects that were not observed in our models using an adenovirus expressing SIRT1. While our studies suggest SIRT1 has the potential to be a beneficial therapeutic target, several more studies are required to demonstrate the safety and efficacy of pharmacological activation of SIRT1 to suppress liver I/R injury.

**Overall Conclusion**

Mitofusin 2 mediates Sirtuin 1 induced autophagy to suppress liver I/R injury.
Figure 6-1. MFN2 mediates SIRT1 induced autophagy to suppress against liver I/R injury. (A) Normal hepatocyte response to I/R. During ischemia, SIRT1 and MFN2 are reduced by cathepsins. Prolonging ischemia activates calpains which may be the lethal event leading to reperfusion injury. Calpain activation depletes SIRT1, but other factors deplete MFN2. Upon reperfusion, SIRT1 remains depleted, while MFN2 expression increase but are subjected to removal through cysteine proteases. Autophagy is dysfunctional leading to the MPT onset and cell death. (B) Activation of SIRT1 enhances autophagy and increases ATG7 expression. During ischemia, the endogenous reduction pathway remains activated but SIRT1 activation leads to heightened levels of MFN2 that are resistant to degradation during reperfusion. Autophagy is functional and the MPT onset is blocked leading to the re-establishment of autophagic flux to sustain viable hepatocytes. (C) Activation of SIRT1 in MFN2 deficient hepatocytes impairs basal autophagy flux leading to the depletion of MFN2 during reperfusion, the MPT onset and hepatocyte death. Collectively, these data show that SIRT1 induced autophagy can suppress liver I/R injury.
Table 6-1. SIRT1 agonists and clinical trials are controversial.

<table>
<thead>
<tr>
<th>Author - year</th>
<th>Participants</th>
<th>Objective</th>
<th>Agonist and Dose</th>
<th>Duration</th>
<th>Brief description of the outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brasnyo – 2011 (365)</td>
<td>Type 2 diabetics</td>
<td>Insulin sensitivity and oxidative status</td>
<td>5 mg - resveratrol</td>
<td>Twice daily – 28 days</td>
<td>Decreased insulin and oxidative stress</td>
</tr>
<tr>
<td>Timmers – 2011 (366)</td>
<td>Obese men</td>
<td>Metabolic changes</td>
<td>75 mg - resveratrol</td>
<td>Twice daily – 30 days</td>
<td>Reduced glucose and insulin, reduced liver fat, improved muscle mitochondrial function</td>
</tr>
<tr>
<td>Wong – 2011 (367)</td>
<td>Obese men and women with hypertension</td>
<td>Flow mediated dilution</td>
<td>30-270 mg -resveratrol</td>
<td>Signal dose</td>
<td>Increase flow mediated dilation</td>
</tr>
<tr>
<td>De Groote – 2012 (368)</td>
<td>Healthy obese men and women</td>
<td>Oxidative stress markers</td>
<td>150 mg resveratrol</td>
<td>Daily – 4 weeks</td>
<td>Increase antioxidant activity by increase redox related genes</td>
</tr>
<tr>
<td>Poulsen – 2013 (369)</td>
<td>Healthy obese men</td>
<td>Metabolic effects</td>
<td>500 mg resveratrol</td>
<td>Three times daily – 4 weeks</td>
<td>No effect on insulin sensitivity, blood pressure, lipid oxidation, and metabolic markers</td>
</tr>
<tr>
<td>Yoshino – 2012 (370)</td>
<td>Non-obese women</td>
<td>Metabolic effects</td>
<td>75 mg - resveratrol</td>
<td>Daily – 12 weeks</td>
<td>No effect on insulin sensitivity to skeletal muscle metabolism</td>
</tr>
<tr>
<td>Libri – 2012 (364)</td>
<td>Healthy elderly men and women</td>
<td>Metabolic effects</td>
<td>0.5 – 2 g –SRT2104</td>
<td>Daily – 28 days</td>
<td>Decrease cholesterol, triglycerides, improved mitochondrial function</td>
</tr>
<tr>
<td>Vekatasubramanian – 2013 (371)</td>
<td>Healthy cigarette smokers</td>
<td>Metabolic effects</td>
<td>2 g – SRT2104</td>
<td>Daily – 28 days</td>
<td>No effect on cardiac rhythm, decreased cholesterol</td>
</tr>
</tbody>
</table>


BIOGRAPHICAL SKETCH

Thomas Biel obtained his Bachelor of Science degree in biology from Keystone College, Pennsylvania (2004-2009) and joined the Interdisciplinary Biomedical Sciences Program at the University Of Florida College of Medicine in 2009. Under the mentorship of Dr. Jae-Sung Kim and Dr. Kevin Behrns, Mr. Biel has presented his research in several international conferences such as the American Association for the Study of Liver Disease (AASLD), Experimental Biology (EB), and Mitochondria and Hepatotoxicity as well as participated in Department of Surgery’s Research Day, and the College of Medicine Research Day. Throughout his training he has received honors and awards such as the AALSD posters of distinction and second place for research accomplishments in the College of Medicine’s graduate student competition. Upon completion of his PhD program in medical sciences in December 2014, he aspires to remain in the area of mitochondrial therapeutics for medicinal treatments.