

THE ROLE OF AUTOPHAGY IN OXIDATIVE STRESS-MEDIATED DYSFUNCTION IN
CARDIAC CELLS AND RODENT HEARTS

By

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To my family for their unconditional love

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LIST OF ABBREVIATIONS

2DG	2-deoxy-d-glucose
3-MA	3-Methyladenine
8-oxo-dGuo	8-oxo-7,8-dihydro-2'-deoxyguanosine
8-oxo-Guo	8-oxo-7,8-dihydroguanosine
AMA	Antimycin A
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
BafA1	Bafilomycin A1
Bcl-2	B cell leukemia-2
Bcl-X _L	B cell leukemia-X long
Bnip3	Bcl-2 and adenovirus E1B 19 kDa-interacting protein-3
CAD	coronary artery disease
CK	creatine kinase
CMA	chaperone-mediated autophagy
CR	calorie restriction
CuZnSOD	copper-zinc-containing superoxide dismutase
CVD	cardiovascular disease
CypD	cyclophilin D
DFOM	deferoxamine mesylate
EGTA	ethylene glycol tetraacetic acid
ETC	electron transport chain
FBN	Fischer 344 x Brown Norway
FIP200	Focal adhesion kinase family interacting protein of 200 kD
FSC	forward scatter

Gpx	glutathione peroxidase
GTC	guanidium thiocyanate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1 α	hypoxia-inducible factor-1 α
HPLC-ECD	HPLC coupled to electrochemical detection
I/R	ischemia/reperfusion
IGF-1	insulin-like growth factor-1
IP ₃	inositol triphosphate
IP ₃ R	inositol triphosphate receptor
LAMP-1	lysosomal membrane-associated protein-1
LAMP-2	lysosomal membrane-associated protein-2
LC3	light chain-3
LDH	lactate dehydrogenase
LVH	left ventricular hypertrophy
MAPKs	mitogen activated protein kinases
mCAT	catalase targeted to the mitochondrial matrix
MnSOD	manganese-containing superoxide dismutase
mPTP	mitochondrial permeability transition pore
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide\
NADH	nicotinamide adenine dinucleotide (reduced)
NAPDH	nicotinamide adenine dinucleotide phosphate
nDNA	nuclear DNA

NF-κB	nuclear factor κB
Nix	Nip3-like protein X
NRF-1	nuclear respiratory factor-1
O ₂ ^{•-}	superoxide radical
OXPHOS	oxidative phosphorylation
PARL	presenilins-associated rhomboid-like
pCAT	peroxisomal catalase
PE	phosphatidylethanolamine
PGC-1α	peroxisome proliferator-activated receptor-γ coactivator-1α
PI3K	phosphatidylinositol-3-kinase
PKB/Akt	protein kinase B
PolG	mtDNA polymerase γ
Prx	peroxiredoxin
Resv	Resveratrol
RHEB	Ras homolog enriched in brain
ROS	reactive oxygen species
SIRT1	Sirtuin 1
SNARE	Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor
SQSTM1	Sequestosome 1
SSC	side scatter
TD-NMR	time domain – nuclear magnetic resonance
TFAM	mitochondrial transcription factor A
TMRM	Tetramethyl Rhodamine Methyl Ester
TNF-α	tumor necrosis factor α
TSC1/TSC2	tuberous sclerosis complex ½

ULK1	unc-51-like kinase 1
UPS	ubiquitin proteasomal system
UBA	ubiquitin associated
UVRAG	UV radiation resistance-associated gene
VDAC	voltage-dependent anion channel
Vps	vacuolar protein sorting
$\Delta\psi_m$	mitochondria membrane potential

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Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in the developing world. While long-term exposure to cardiovascular risk factors plays a major role in the etiopathogenesis of CVD, oxidative stress-induced cardiac damage enhances the susceptibility to develop heart pathologies in late life. The enhanced generation of reactive oxygen species, especially by damaged mitochondria, is considered to be a major contributing mechanism. Hence, the removal of oxidatively modified cellular components and dysfunctional mitochondria is critical for maintenance of cellular homeostasis.

Autophagy is a self-digestion process which can degrade damaged proteins and organelles and is also the only known mechanism targeting dysfunctional mitochondria for removal. In our studies, we have investigated whether pharmacological or nutritional enhancement of autophagy offers protection against oxidative stress-mediated dysfunction in a cardiomyocyte cell line (HL-1) and in late middle-aged rat hearts. In HL-1 cells, we mimicked mitochondria-mediated oxidative stress by treating cells with Antimycin A (AMA). AMA increased mitochondrial superoxide generation, decreased

mitochondrial membrane potential, increased nuclear DNA oxidation and decreased cellular respiration. Treatment of HL-1 cells with the mTOR inhibitor rapamycin lead to a strong induction of autophagy and mitophagy and protected against the cytotoxic effects of AMA, assessed by cell survival and apoptotic signaling analysis. Rapamycin also prevented AMA-mediated increases in ubiquitinated protein aggregates. Autophagy inhibition attenuated the cytoprotective effects of rapamycin. For our in vivo experiments, we investigated whether a late-life intervention of moderate calorie restriction (CR, 20%) alone, or in combination with the plant polyphenol resveratrol can induce autophagy in the hearts of late middle-aged rats. We further investigated whether such an induction of autophagy is protective against the cardiotoxic effects of the chemotherapeutic drug doxorubicin, a known oxidative stressor. Our observed that CR and resveratrol, only when combined, stimulated autophagic flux in the left ventricular tissue of late middle-aged rat hearts. Additionally, autophagy induction protected against the cytotoxic effects of doxorubicin, as assessed by apoptotic analysis in the myocardium and cardiac damage markers in serum. Our studies therefore suggest that interventions aimed at enhancing basal autophagy may offer protection against oxidative stress-mediated dysfunction in cardiomyocytes.

CHAPTER 1 INTRODUCTION

Oxidative Stress and Mitochondria in Cardiovascular Diseases

The worldwide trend in cardiovascular diseases (CVDs) has been growing at an alarming rate and it is estimated that by the year 2020, up to 40% cases of deaths would be due to cardiovascular complications (Braunwald, 1997). In the United States alone, mortality data from 2008 shows that 1 of every 3 deaths results from CVD (Roger et al., 2012). CVD comprises any pathologic changes affecting the heart itself or the vessels carrying blood to and from the heart. CVD includes, but is not limited to heart failure, ischemic heart disease, left ventricular hypertrophy (LVH), coronary artery disease (CAD), hypertensive cardiomyopathy and diabetic cardiomyopathy. Notably, the prevalence of such cardiac pathologies increases with age. The disproportionate occurrence of CVD at advanced age is largely attributable to long-term exposure to cardiovascular risk factors such as hypertension, dyslipidemia, diabetes mellitus, physical inactivity, etc (Kovacic et al., 2012). In addition, oxidative stress-mediated damage observed during intrinsic cardiac aging can cause structural and functional alterations in the heart and render it more vulnerable to various stressors, ultimately favoring the development of CVD (Lakatta, 2001). Reactive oxygen species (ROS) are constantly generated within cells by several enzymatic reactions, including those catalyzed by cyclooxygenases, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase (Paravicini & Touyz, 2008). However, the bulk of ROS production occurs as a byproduct of mitochondrial oxidative phosphorylation (OXPHOS). Experimental evidence indicates that mitochondrial function decreases over the course of aging, resulting in increased ROS generation, enhanced free radical-

inflicted damage and further mitochondrial decay (Balaban et al., 2005). In this scenario, the removal of dysfunctional mitochondria and oxidatively damaged components generated thereof, through the self-digestion process of autophagy is critical for the maintenance of cell viability (Levine & Klionsky, 2004). The efficiency of this process declines with advancing age which may be critically involved in heart senescence as well as in age-related CVD (Inuzuka et al., 2009; Taneike et al., 2010)

In this chapter, we will highlight the role played by oxidative stress, especially those generated by the mitochondria, in causing cardiac senescence and predisposing it to age-related CVD. This chapter will also illustrate the putative mechanisms whereby dysfunctional autophagy can cause pathologic changes in the heart and play a role in the pathogenesis of specific heart diseases especially prevalent in late life. Interventions proposed to counter cardiac oxidative stress through improvements in autophagy are also presented, along with a general discussion of our research goals of using autophagy as a therapeutic intervention against CVD.

Mechanisms of Cardiac Mitochondrial Dysfunction

Mitochondria are essential for cardiomyocyte function and viability. Indeed, the myocardium is a highly energy demanding tissue, with mitochondria supplying over 90% of ATP. Free radicals are constantly generated during mitochondrial respiration. Under physiological conditions, 0.2-2% of oxygen is converted into superoxide anion ($O_2^{\cdot-}$) mainly at complex I and III of the electron transport chain (ETC) (Boveris & Chance, 1973). To counteract the burden of ROS production, the mitochondrion is equipped with a multileveled defense network comprising detoxifying enzymes and non-enzymatic antioxidants (Andreyev et al., 2005a). Within the mitochondrial matrix, manganese-containing superoxide dismutase (MnSOD, SOD2) converts $O_2^{\cdot-}$ into hydrogen peroxide

(H₂O₂), which is further detoxified into O₂ and H₂O by glutathione peroxidase (Gpx-I) and peroxiredoxine (Prx-III). Alternatively, O₂^{•-} can be released in the mitochondrial intermembrane space where it is converted to H₂O₂ by copper-zinc-containing SOD (CuZnSOD, SOD1). In addition, O₂^{•-} leaked in the intermembrane space can be scavenged by cytochrome *c* (Pasdois et al., 2011).

Once merely considered unwanted byproducts of OXPHOS, small amounts of oxidants have now been shown to function as essential signaling molecules, necessary for the induction of endogenous defense mechanisms that culminate in increased stress resistance (Finkel, 2011). In contrast, the generation of excessive ROS by dysfunctional mitochondria, often coupled with a defective oxidant scavenging, have been implicated in the aging process as well as in the pathogenesis of several chronic degenerative diseases, including CVD (Judge & Leeuwenburgh, 2007).

Damaged cardiac mitochondria can release up to 10-fold more H₂O₂ than intact organelles (Grivennikova et al., 2010). Furthermore, in the presence of non-protein-bound redox cycling metals (e.g., iron and copper), H₂O₂ can be converted into the highly reactive hydroxyl radical (•OH), through Fenton's and Haber-Weiss' reactions. In such circumstances, the mitochondrion is exposed to a high burden of oxidative stress, resulting in the primary damage to its own constituents. It is worth mentioning that the mitochondrial iron content increases with aging in rodent post-mitotic tissues, including the myocardium, which may exacerbate the extent of oxidative damage in late life (Xu et al., 2010).

The mitochondrial DNA (mtDNA) is especially prone to oxidative damage due to its proximity to the ETC, the lack of protective histones and a less efficient repair system

compared with nuclear DNA (nDNA) (Yakes & Van, 1997). As a result, the level of oxidatively-modified bases in mtDNA is several-fold higher than that in nDNA (Yakes & Van, 1997). Moreover, due to the compactness of the mitochondrial genome (i.e., lack of introns), each mutation is likely to affect gene integrity and hence, mitochondrially encoded protein, mRNA and tRNA function (Wei & Lee, 2002). It follows that mtDNA mutations can lead to the synthesis of defective ETC components, resulting in impairment of OXPHOS, decreased ATP production and further ROS generation (Linnane et al., 1989). The vicious cycle originating from ROS-inflicted mtDNA damage represents the main tenet of the mitochondrial free radical theory of aging and is also believed to play a central role in the pathogenesis of age-associated degenerative diseases, including CVD (Linnane et al., 1989).

Consequences of Abnormal Mitochondrial Free Radical Generation in the Heart

Elevated levels of oxidative damage to mitochondrial proteins, lipids and nucleic acids have been detected in the failing myocardium of old rodents (Barja & Herrero, 2000; Judge et al., 2005b; Leeuwenburgh et al., 1997). The frequency of mtDNA point mutations and deletions is ~3-fold higher in the aged mouse heart compared with young adult controls (Dai & Rabinovitch, 2009). Similarly, the frequency of the common 4977 bp deletion of mtDNA increases during aging in the human heart, and is 5 to 15-fold higher in persons over 40 years of age than in younger individuals (Liu et al., 1998; Mohamed et al., 2006). However, the proof of principle that the accumulation of mtDNA damage and subsequent mitochondrial dysfunction may be causative to cardiac senescence has been provided by the characterization of mice expressing a proofreading-deficient mtDNA polymerase γ (PolG) (Kujoth et al., 2005; Trifunovic et al., 2004). These mutants accumulate a high load of mtDNA mutations and deletions, and

are characterized by the early appearance of cardiac pathologic changes such as cardiac enlargement. Heart mitochondria of PolG mice exhibit abnormal ETC with depressed activity of complex I and IV, reduced ATP production, and accumulation of enlarged, irregularly shaped mitochondria (Trifunovic et al., 2004). Furthermore, levels of protein carbonyls are increased in cardiac mitochondria from mtDNA mutator mice compared with wild-type rodents (Dai et al., 2010). PolG mice die prematurely with dilated cardiomyopathy. Severe cardiomyopathy has also been observed in mice expressing a heart-specific proofreading-deficient mtDNA polymerase (Zhang et al., 2003). Remarkably, the PolG heart phenotype, the cardiac mtDNA mutation load and the extent of mitochondrial protein oxidation are partially rescued by the overexpression of catalase targeted to the mitochondrial matrix (mCAT) (Dai et al., 2010). In addition, mCAT overexpression extends mean and maximum lifespan and delays the development of cardiac pathology in mice (Dai et al., 2009a; Schriener et al., 2005). The extent of mitochondrial oxidative damage, including mtDNA deletions, and the rate of H₂O₂ generation, are significantly attenuated in the heart of old mCAT mice compared with age-matched wild-type littermates (Dai et al., 2009a; Schriener et al., 2005).

In addition to increased ROS generation, dysfunctional mitochondria could lead to the induction of apoptosis through the release of pro-apoptotic factors (Hamacher-Brady et al., 2006; Sybers et al., 1976). Indeed, mitochondria are a major check-point for the integration of apoptotic stimuli. Notably, cardiomyocyte removal through apoptosis increases with advancing age, which, combined with insufficient replenishment by cardiac stem cells, may contribute to the age-related heart remodeling (Marzetti et al., 2009). However, whether the increased severity of apoptosis suffered by the aged

myocardium is directly attributable to autophagic failure is yet to be established. It is therefore reasonable to say that, the efficient removal of damaged and potentially harmful mitochondria along with other oxidatively damaged proteins are vital for the preservation of cardiomyocyte homeostasis.

Autophagic Machinery and Cardiomyocyte Homeostasis

Regardless of the mechanism(s) primarily responsible for enhanced oxidative stress and mitochondrial dysfunction, cellular quality control is essential for the preservation of cardiomyocyte homeostasis. This task is accomplished through the complex coordination of several processes (reviewed by (Tatsuta & Langer, 2008). For instance, oxidatively damaged and misfolded proteins are marked by ubiquitination, to be subsequently removed by the ubiquitin-proteasomal system (Shang & Taylor, 2011). With regard to the mitochondria, an intramitochondrial proteolytic system selectively removes damaged mitochondrial proteins. A second line of defense is provided by the dynamic nature of the mitochondrial population, i.e. the functionality of damaged mitochondria can be restored by their fusion with neighboring, intact organelles (Chen & Chan, 2009). Finally, severely damaged mitochondria and oxidatively modified protein aggregates can be eliminated through autophagy.

Types of Autophagy

Autophagy is a self-eating process through which cells degrade their own components, recycling amino acids and other building blocks that can be eventually reutilized (Yorimitsu & Klionsky, 2005). Such degradation is performed by lysosomal acid hydrolases. Depending on the pathway cellular components are delivered to lysosomes, three types of autophagy can be distinguished: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy

(hereafter simply referred to as autophagy) involves the degradation of long-lived proteins and whole cellular organelles through a multistep process (Fig. 1-1) (Yorimitsu & Klionsky, 2005). It begins with the formation of a double-layered isolation membrane (phagophore) around the molecules and/or organelles to be degraded. The phagophore engulfs and seals around the content, forming an autophagosome. Eventually, the autophagosome fuses with a lysosome, evolving into an autophagolysosome (or autolysosome), wherein lysosomal hydrolases digest the engulfed components (Yorimitsu & Klionsky, 2005). Microautophagy involves the direct sequestration of cytosolic components through invaginations or arm-like projections of the lysosomal membrane (Mijaljica et al., 2011). Microautophagy may serve for the turnover of long-lived proteins; however, the significance and regulation of this type of autophagy in the mammalian system remain poorly understood (Mijaljica et al., 2011). Finally, CMA is a highly selective process devoted to the degradation of soluble cytosolic proteins with specific peptide sequences (Arias & Cuervo, 2011).

Starvation is the strongest stimulator of autophagy (Mizushima et al., 2004; Mortimore & Schworer, 1977). During nutrient deprivation, autophagy breaks down cellular components generating amino acids, fatty acids and carbohydrates, which can be harnessed for energy production as well as for the synthesis of essential cellular molecules. Autophagy is also involved in specific cytosolic rearrangements during embryogenesis and postnatal development (Cecconi & Levine, 2008) and is also induced during viral or bacterial infections (Levine et al., 2011). Furthermore enhanced autophagy has been observed during hypoxia (Zhang et al., 2008) and under various stress conditions, including radiation exposure and increased ROS generation (Paglin et

al., 2001; Scherz-Shouval et al., 2007b). In these circumstances, autophagy is essential for the maintenance of cell homeostasis by promoting the removal of damaged components (Levine & Klionsky, 2004; Levine & Kroemer, 2008). Indeed, impairments in autophagy induce premature aging and shorten the lifespan in several organisms (Hars et al., 2007; Matecic et al., 2010). Conversely, up-regulation of autophagy is proposed to be a major mechanism underlying the lifespan-extending properties of calorie restriction (CR) (Morselli et al., 2010; Toth et al., 2008).

The execution of autophagy involves the coordination of a complex molecular machinery which is briefly described in the next subsection.

Autophagic Process and Molecular Regulation

To date, over 35 AuTophagy-related (Atg) proteins have been identified in yeasts and mammals; however, the precise role each Atg protein plays during autophagy is not fully established (Chen & Klionsky, 2011). As illustrated in Fig. 1-1, the process of autophagy can be divided into discrete steps, namely, induction and nucleation, expansion, fusion, and degradation. The induction phase is mediated by the ULK1-Atg13-FIP200 kinase complex (Xie & Klionsky, 2007). The regulation of the nucleation stage, which consists in the recruitment of Atg proteins to the phagophore assembly site, is not yet completely understood. However, the vacuolar protein sorting-34 (Vps34), a class III phosphatidylinositol-3-kinase (PI3K), is required for this step (Suzuki et al., 2001). Vps34 associates with Beclin1, the mammalian homologue of yeast Atg6, and subsequently recruits Atg14 and Vps15 (p150) to the pre-autophagosomal structure (Suzuki et al., 2001). The elongation and expansion of the phagophore membrane require two ubiquitin-like conjugation systems involving Atg12 (conjugated to Atg5) and Atg8/light chain-3 (LC3, conjugated to phosphatidyl ethanolamine), along with other Atg

proteins such as Atg9 and Atg16 (Xie & Klionsky, 2007). The fusion of the autophagosome with a lysosome relies on the canonical cellular fusion machinery consisting of the Rab-SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptor) system and requires the presence of lysosomal membrane-associated protein-2 (LAMP-2) and the UV radiation resistance-associated gene (UVRAG) (Nair et al., 2011; Tong et al., 2010). Finally, the digestion of the cargo is carried out by lysosomal hydrolases, followed by the transportation of degraded components into the cytoplasm by lysosomal efflux transporters such as Atg22 (Tong et al., 2010).

With regard to the regulation of autophagy, the mammalian target of rapamycin (mTOR) is considered to be a major player, linking the cellular nutritional state with the level of ongoing autophagy (Fig. 1-2) (Kim et al., 2011). Under nutrient rich conditions, mTOR is active and inhibits the ULK1-Atg13-FIP200 complex required for the induction of autophagy (Jung et al., 2009a). Energy deprivation leads to mTOR inactivation, thereby inducing autophagy (Kim et al., 2011; Talloczy et al., 2002). In addition, starvation causes an increase in the cellular AMP:ATP ratio, leading to the activation of AMP-activated protein kinase (AMPK). AMPK in turn promotes autophagy by directly activating ULK1 as well as by relieving the mTOR-mediated inhibition of autophagy (Kim et al., 2011) (Fig.1-2). It is worth mentioning here that in addition to stimulating mitochondrial removal through autophagy, AMPK enhances the activity of sirtuin-1 (SIRT1) and its downstream target PGC-1 α , resulting in the stimulation of mitochondrial biogenesis (Canto et al., 2009). Hence, it is believed that through the activity of AMPK,

mitophagy and mitochondrial biogenesis are coordinately regulated, maintaining a healthy and functional pool of mitochondria in the cell (Fig. 1-2).

Although autophagy might seem to be a random, bulk digestion process, evidence is accumulating that intracellular components can be selectively targeted for degradation (Kadandale & Kiger, 2010). For instance, autophagy can be specifically directed towards the removal of peroxisomes (pexophagy), endoplasmic reticulum (reticulophagy), and ribosomes (ribophagy) (Kadandale & Kiger, 2010). Likewise, mitochondria can be selectively targeted for degradation via mitophagy (Wang & Klionsky, 2011). The currently known molecular machinery and the regulation of this cellular pathway are outlined in the next subsection.

Mitophagy: A Specialized Form of Autophagy

Mitophagy is a highly selective process that can promote the elimination of dysfunctional or unnecessary mitochondria (Wang & Klionsky, 2011). The loss of mitochondrial membrane potential ($\Delta\psi_m$) represents a major trigger of mitophagy (Wang & Klionsky, 2011). Indeed, laser-induced photo-damage of selected mitochondria inside living hepatocytes results in the rapid dissipation of $\Delta\psi_m$, followed by the quick removal of depolarized mitochondria through mitophagy (Kim & Lemasters, 2011). In addition, oxidative damage can lead to the formation of asymmetric daughter mitochondria characterized by different $\Delta\psi_m$, with autophagy specifically targeting mitochondria with lower $\Delta\psi_m$ (Twig et al., 2008). Apart from the degradation of damaged mitochondria under stress conditions, mitophagy is essential for mitochondrial turnover in the basal state as well as during cell differentiation, such as the maturation of reticulocytes into

mature red blood cells (Tal et al., 2007). The occurrence of selective mitophagy in cardiomyocytes has not yet been conclusively demonstrated.

Investigations into the molecular regulation of mitophagy have unveiled several mitophagy-specific proteins (Green et al., 2011). Parkin and Pink1 are believed to play an important role in the selective degradation of damaged mitochondria, at least under certain circumstances (Narendra et al., 2008). Parkin is a cytosolic E3-ubiquitin ligase which is selectively recruited to dysfunctional mitochondria, and assists in their removal by mitophagy (Narendra et al., 2008). Pink1 is imported into healthy mitochondria through a $\Delta\psi_m$ -dependent process and is degraded by the presenilins-associated rhomboid-like (PARL) protease (Matsuda et al., 2010). The dissipation of $\Delta\psi_m$ results in the accumulation of Pink1 on the mitochondrial surface, leading to the recruitment of Parkin, which ubiquitinates outer membrane proteins including the voltage-dependent anion channel (VDAC) (Geisler et al., 2010). It is proposed that ubiquitin-tagged mitochondria are directly targeted to autophagic vacuoles through the interaction of ubiquitinated proteins with the autophagosomal marker LC3, mediated by the adapter protein p62 (Geisler et al., 2010). In addition, Parkin can ubiquitinate B cell leukemia-2 (Bcl-2), therefore derepressing Beclin1 and activating autophagy (Chen et al., 2010).

Recent evidence also suggests that the opening of the mitochondrial permeability transition pore (mPTP) may be required for the selective removal of damaged mitochondria (Carreira et al., 2010; Elmore et al., 2001). Opening of the mPTP causes a sudden increase of the inner membrane permeability to solutes with molecular weight up to 1,500 Da (Weiss et al., 2003). This results in mitochondrial depolarization, activation of the mitochondrial F_0F_1 ATPase (i.e., ATP synthase operating in reverse),

and swelling and rupture of the outer membrane (Weiss et al., 2003). The loss of $\Delta\Psi_m$ subsequent to permeability transition targets individual mitochondria for degradation (Carreira et al., 2010). Notably, in cultured cardiomyocytes, starvation-induced autophagy is preceded by mitochondrial depolarization (Carreira et al., 2010). The loss of $\Delta\Psi_m$ and the activation of autophagy are prevented by cyclosporin A, an inhibitor of the mPTP component cyclophilin D (CypD) (Carreira et al., 2010). Furthermore, starvation fails to induce autophagy in CypD-deficient murine cardiomyocytes, whereas in cardiac cells from mice overexpressing CypD autophagy is enhanced even under fed conditions (Carreira et al., 2010). The NAD-dependent deacetylase sirtuin-3 (SIRT3) appears to be critically involved in the control of mPTP by modulating CypD (Hafner et al., 2010). Indeed, in transgenic mice, the loss of SIRT3 activity leads to increased activation of the mPTP in cardiac mitochondria in response to Ca^{2+} increases and hemodynamic stress, the latter commonly observed in CVD (Hafner et al., 2010).

Similar to the mPTP, the apoptotic proteins Bnip3 (Bcl-2 and adenovirus E1B 19 kDa-interacting protein-3) and Nix (Nip3-like protein X) are thought to trigger selective mitophagy through mitochondrial depolarization (Zhang & Ney, 2009). Moreover, Bnip3 may induce mitophagy by competitively disrupting the inhibitory interaction between Bcl-2 and Beclin-1 (Zhang & Ney, 2009). Finally, Nix associates with mitochondrial membranes and directly interacts with LC3 (Novak et al., 2010).

Cross-talk between Autophagy and the Ubiquitin-Proteasomal System for Protein Quality Control

The ubiquitin-proteasomal system (UPS) provides to the selective elimination of short-lived and misfolded proteins, small enough to enter the narrow barrel-shaped proteasome for digestion (Schrader et al., 2009). Proteins are targeted for degradation

by covalent binding to ubiquitin chains. Individual ubiquitin molecules are activated by ubiquitin-activating enzymes (E1s) and transferred to ubiquitin-conjugating enzymes (E2s). Afterwards, ubiquitin is transferred to the substrate, which is recognized by ubiquitin-protein ligases (E3s). Polyubiquitin chains function as recognition motifs for delivery to 26S proteasomes where the hydrolysis takes place (Schrader et al., 2009). Alternatively, misfolded monomeric proteins may be degraded by CMA independent of polyubiquitination (Arias & Cuervo, 2011). However, if the degradative capacity of the UPS and CMA is overwhelmed, misfolded proteins accumulate, forming large aggregates, known as inclusion bodies and aggresomes (Markossian & Kurganov, 2004). The formation of protein aggregates is promoted by the ubiquitin-binding protein p62/SQSTM1 (sequestosome 1) and NBR1 (neighbor of BRCA1 gene 1). Such protein aggregates are cleared through autophagy (Zheng et al., 2011). The existence of a cross-talk between the UPS and autophagic system is witnessed by the observation that inhibition of proteasomal degradation increases the appearance of protein aggregates within autophagic vacuoles (Wojcik et al., 1996). Moreover, inhibition of autophagy due to dynein mutations leads to the accumulation of cytosolic protein aggregates in mouse neurons (Ravikumar et al., 2005). Importantly, p62/SQSTM1 is required for the selective autophagic removal of protein aggregates. p62 binds to ubiquitinated proteins through its ubiquitin-associated (UBA) domain (Seibenhener et al., 2004) and interacts with Atg8/LC3 through the LC3 recognition sequence (LRS) (Noda et al., 2008). This establishes a physical link between the protein aggregates and the autophagosomal system, thereby mediating their clearance.

Impact of Impaired Autophagy: Accumulation of Dysfunctional Mitochondria and Lipofuscin

Cardiomyocytes are terminally differentiated, post-mitotic cells with a lifespan of several decades. Hence, the maintenance of a healthy pool of mitochondria and the efficient removal of damaged and potentially harmful organelles are vital for the preservation of cardiomyocyte homeostasis. A decrease in autophagic flux may result in the accumulation within cardiomyocytes of dysfunctional mitochondria that are bioenergetically inefficient and prone to ROS leakage (Terman et al., 2010). Importantly, autophagic flux has been shown to be decreased with age (Inuzuka et al., 2009; Taneike et al., 2010) and the ultrastructural analysis of myocardium from aged rodents has revealed the presence of enlarged mitochondria, characterized by swelling, loss of cristae and matrix derangement (Sachs et al., 1977b; Vanneste & van den Bosch de, 1981). Biochemically, these senescent mitochondria exhibit reduced ATP production and increased ROS generation (Karbowski et al., 1999). It is hypothesized that giant mitochondria may progressively displace functional ones (Brunk & Terman, 2002), attributed to a replicative advantage of damaged mitochondria, secondary to their partially deleted genome (Arnheim & Cortopassi, 1992). Alternatively, giant mitochondria may benefit from a survival advantage, being less likely to be autophagocytosed by virtue of their dimensions. Along these lines, the so-called survival of the slowest (SOS) theory postulates that damaged mitochondria would suffer from less ROS damage on their own membranes due to a reduced respiratory function, and would consequently be less targeted for autophagy compared with intact mitochondria (de Grey, 1997).

Importantly, oxidative stress is associated with the accumulation within post-mitotic cells of a non-degradable, polymeric, toxic, yellow-brown pigment, called lipofuscin or age pigment (Jung et al., 2007). Lipofuscigenesis results from peroxide-induced Fenton reactions elicited by intralysosomal materials producing highly reactive hydroxyl radicals (Fig. 1-3). ROS-derived modifications to proteins and lipids cause crosslinking inside lysosomes/autolysosomes, generating lipofuscin (Jung et al., 2007). Peroxides involved in these Fenton reactions can diffuse into lysosomes from cytosolic damaged mitochondria or may originate from autophagocytosed, yet undegraded mitochondria (Terman & Brunk, 2005). The accumulation of such intracellular garbage eventually overburdens the autophagosomal-lysosomal degradative capacity, by acting as a sink for lysosomal hydrolases (Terman, 2001). It follows that attempts to digest lipofuscin eventually results in the incapacity of autophagy to keep up with the cell's needs (Terman, 2001). This series of events is thought to trigger a vicious cycle in which the autophagic failure and the accumulation of damaged mitochondria perpetuate each other, resulting in further oxidative stress and enhanced lipofuscinogenesis (Terman & Brunk, 2005). The collapse of the catabolic machinery will eventually become incompatible with the maintenance of cell homeostasis and survival. This assumption also represents the basis of the mitochondrial-lysosomal axis theory of aging (Terman, 2001; Terman et al., 2010).

Mitochondrial dysfunction and oxidative stress is implicated in the pathogenesis of a host of heart diseases highly prevalent at old age, including heart failure, LVH, heart attack and diabetic cardiomyopathy (Lesnefsky et al., 2001). It is worth noting that an altered regulation of autophagy has been shown to contribute to the pathogenesis of

these conditions, further supporting the relevance of the mitochondrial-lysosomal axis to cardiac physiology. In the following sections, the role played by cardiomyocyte mitochondrial dysfunction and abnormal regulation of autophagy in the above mentioned heart diseases is discussed.

Heart Failure

Various stressors, as well as pressure overload may be responsible for mitochondrial dysfunction and enhanced ROS generation during heart failure (Tsutsui et al., 2009). Heart failure mitochondria generate larger amounts of $O_2^{\bullet-}$ compared with normal mitochondria (Ide et al., 2001). This enhanced oxidant production has been associated with increased levels of lipid peroxidation to mitochondrial membranes, decreased mtDNA copy number, reduced abundance of mitochondrial RNA transcripts, and impaired ATP generation capacity (Ide et al., 2001). In addition, oxidative stress directly impacts cardiomyocyte structure and function by activating signaling pathways involved in myocardial remodeling and heart failure (Ide et al., 2001; Spinale et al., 1998). This suggests the existence of a pathogenic link between enhanced ROS production, mitochondrial dysfunction and the development of heart failure. Recent evidence also suggests that an altered regulation of cellular quality control by autophagy, may contribute to the pathogenesis of heart failure. For instance, cardiomyocytes isolated from autophagy deficient mice show an increased sensitivity to β -adrenergic stress compared with wild-type cells (Nakai et al., 2007b). Indeed, stress induced by 7-day isoproterenol treatment resulted in left ventricular dilation and cardiac dysfunction in autophagy deficient mice, but not in wild-type controls (Nakai et al., 2007b).

Left Ventricular Hypertrophy

Mitochondrial dysfunction is implicated in the pathogenesis of left ventricular hypertrophy as well as in the transition from compensated left ventricular hypertrophy to heart failure (Abel & Doenst, 2011). Mitochondrial misalignment and aggregation are observed in adult mice with heart-specific deficiency of autophagy in response to pressure overload induced by thoracic transverse aortic constriction (Nakai et al., 2007b). These animals develop LVH, contractile dysfunction and heart dilation. Such findings suggest that an abnormal regulation of autophagy may contribute to the development of left ventricular hypertrophy, possibly through the accumulation of dysfunctional mitochondria and subsequent increased ROS generation. In another study, four weeks of 40% CR increased the activation of cardiomyocyte autophagy and mitigated heart macroscopic and ultrastructural remodeling, while reducing mortality (Finckenberg et al., 2012).

Heart Attack

Oxidative stress plays a central role in the pathogenesis of myocardial damage during oxygen deprivation observed during heart attack. In this setting, mitochondria contribute to cardiac dysfunction and cardiomyocyte injury via both a loss of metabolic function and an increased generation of oxidants (Lesnefsky et al., 2001). Oxidative stress is further exacerbated by the concomitant impairment of endogenous antioxidant defenses (Becker, 2004). Autophagy is activated in response to myocardial oxygen deprivation and promotes cardiomyocyte survival, likely via maintaining energy production during acute nutrient deprivation (Matsui et al., 2007). The degradation of proteins and organelles by autophagy generates amino acids and fatty acids, which can

be used to maintain mitochondrial ATP production and promote survival of cardiac cells (Loos et al., 2011).

Diabetic Cardiomyopathy

The inefficient autophagic removal of damaged mitochondria may promote the accumulation of dysfunctional organelles in the diabetic heart. Indeed, down-regulation of cardiac autophagy, secondary to reduced AMPK activity, has been documented in diabetic mice (Xie et al., 2011b). Ultrastructurally, hearts from these rodents display several morphological aberrations, including aggregation of chaotically distributed mitochondria (Xie et al., 2011b). Inhibition of AMPK by a cardiac-specific dominant negative AMPK gene further reduced autophagy, exacerbated ultrastructural aberrations, worsened cardiac dysfunction and increased mortality in diabetic mice (Xie et al., 2011b). In contrast, treatment with the AMPK-activator metformin significantly enhanced autophagy and ameliorated cardiomyocyte ultrastructural abnormalities, while preserving cardiac function. Such benefits were not observed in rodents expressing a dominant negative AMPK, indicating that cardioprotection by metformin is accomplished through AMPK-mediated up-regulation of autophagy (Xie et al., 2011b).

Collectively, these findings suggest that depression of autophagy may promote the accumulation of dysfunctional mitochondria in the diabetic myocardium, thereby contributing to the development of diabetic cardiomyopathy. Therefore, interventions that up-regulate autophagy appear as promising means to prevent and/or treat this relevant complication of diabetes.

It is however worth remembering that in all the examples of cardiac pathology above, the level of autophagy may be critical in determining whether autophagy will be protective or detrimental. Indeed, the up-regulation of autophagy might represent an

attempt to cope with increased levels of mitochondrial dysfunction and cellular damage. However, the possibility exists that an excessive up-regulation of autophagy is detrimental, chewing up essential survival components in the failing myocardium and leading to further toxicity.

Autophagy as a Therapeutic Target against Cardiac Pathology

The critical role postulated for mitochondria-driven oxidative damage in cardiac aging and CVD would suggest that the administration of antioxidants might mitigate the burden of cardiomyocyte injury. However, the efficacy of antioxidant supplementation is still a matter of debate. Indeed, most clinical trials failed to show any positive effect of antioxidants on cardiovascular outcomes (Bjelakovic et al., 2007). Chronic administration of β -carotene, vitamin A or vitamin E may even increase cardiovascular mortality (Bjelakovic et al., 2007). Exploiting the ability of cells to repair or replace oxidatively-damaged molecules and mitochondria represents an appealing alternative against heart senescence and associated pathologies (Rubinsztein et al., 2011). A schematic of the potential beneficial effects of autophagy against oxidative-stress mediated cellular and mitochondrial dysfunction is shown in Figure 1-4. Interventions aimed at improving mitochondrial turnover through the fine-tuning of autophagy (e.g., CR, resveratrol administration and sirtuin pathway activation) might be especially relevant to prevent age-related CVD (Green et al., 2011).

CR, defined as a reduction in food intake without malnutrition, is the most robust intervention for improving health, maintaining organ function and increasing mean and maximum life span in a range of species (Omodei & Fontana, 2011). Wohlgemuth et al.... showed that lifelong 40% CR increased the protein expression of Atg7, Atg9 and

lipidated LC3 (LC3-II) in the heart of old rats (Wohlgemuth et al., 2007). More recently, Shinmura et al... demonstrated that a similar dietary regimen enhanced the autophagic flux in the heart of aged rats through mTOR suppression (Shinmura et al., 2011). The modulation of the autophagic response represents a primary mechanism underlying the lifespan-extending properties of CR (Hars et al., 2007; Morselli et al., 2010; Toth et al., 2008). Indeed, the inhibition of autophagy prevents the anti-aging effects of CR in lower organisms (Jia & Levine, 2007). CR can induce autophagy through different pathways: the insulin-like growth factor-1 (IGF-1) / insulin signaling pathway (Toth et al., 2008), the sirtuin pathway (Morselli et al., 2010), the AMPK pathway (Egan et al., 2011) and the mTOR pathway (Kim et al., 2011). These different pathways are intimately interconnected and all play important roles in mediating different aspects of the response. These adaptations were associated with reduced lipofuscin accumulation in the myocardium, down-regulation of cardiomyocyte apoptosis, decreases in fiber cross-sectional area, and preservation of left ventricular diastolic function (Shinmura et al., 2011). Similar echocardiographic findings have been reported in late-middle aged humans on long-term CR (Meyer et al., 2006). However, whether these effects were linked with changes in autophagy activity was not investigated. Furthermore, it is presently unclear if the cardioprotective effects of CR are primarily mediated by improvements in autophagy.

Considerable effort has also been directed toward the discovery of drugs that could mimic the effects of CR, without requiring food restriction and its detrimental consequences (Ingram et al., 2006). The first CR-mimetic identified was 2-deoxy-D-glucose (2DG), an analog of glucose, shown to extend both mean and maximum

lifespan in *C. elegans* (Schulz et al., 2007). However, a recent study demonstrated that chronic 2DG administration to rats, although reproducing a CR-like phenotype, caused cardiotoxicity and increased mortality (Minor et al., 2010). Promising CR-mimetics with autophagy-inducing properties are those that intersect with the critical signaling pathways identified above and include biguanides, such as metformin that targets the AMPK and insulin signaling pathways (Xie et al., 2011a), resveratrol that affects sirtuin activity (Morselli et al., 2010) and rapamycin that interacts with mTOR signaling (Harrison et al., 2009).

Resveratrol has been shown to recapitulate the transcriptional profile and some of the physiological changes that develop under CR (Barger et al., 2008a; Barger et al., 2008b; Baur et al., 2006). In addition, resveratrol improved survival and reduced the prevalence of cardiac pathology in mice fed a high-calorie diet (Baur et al., 2006). Studies in rodents have also shown that resveratrol inhibits cardiomyocyte apoptosis, protects the myocardium against I/R injury, prevents LVH, improves endothelial function, inhibits platelet aggregation, and reduces inflammation (Petrovski et al., 2011). SIRT1, which is directly or indirectly activated by resveratrol, exerts a similar hormetic action on cardiomyocyte physiology (Alcendor et al., 2007). Low (2.5-fold) to moderate (7.5-fold) transgenic overexpression of SIRT1 in the mouse heart attenuates age-dependent hypertrophy and reduces the severity of apoptosis/fibrosis as well as cardiac dysfunction (Alcendor et al., 2007). In contrast, high levels (12.5-fold) of SIRT1 expression increase the extent of cardiomyocyte apoptosis and the degree of hypertrophy, while decreasing cardiac function, thereby inducing the development of cardiomyopathy (Alcendor et al., 2007). Importantly, resveratrol has been shown to

induce autophagy in numerous cell lines from different tissues of origin (Jeong et al., 2012; Lv & Zhou, 2012; Mauthe et al., 2011; Wu et al., 2011). Whether resveratrol also induces autophagy in animal models is yet to be determined.

In conclusion, interventions aimed at modulating autophagy may represent a novel strategy to prevent oxidative stress-associated CVD. However, a deeper understanding of the role of autophagy in heart physiology is necessary to determine the “therapeutic amount of stress” and the “hormetic window” that elicit an autophagic response within the adaptive range.

Summary and Project Goals

Over their lifespan, cardiac cells suffer from a high burden of mitochondria-derived oxidative damage, which cannot be diluted through cell proliferation. This implies that the maintenance of a healthy pool of mitochondria and the removal of damaged organelles are vital for the preservation of cardiomyocyte function and viability. Autophagy serves this essential homeostatic function. In fact, the vital functions carried out by autophagy in cardiac physiology suggest the possibility that therapeutic interventions targeting this cellular pathway may represent effective means to counter oxidative stress-induced cardiomyopathy. Hence, we report the use of both in vitro and in vivo systems to investigate whether enhanced autophagy can provide protection against oxidative stress-mediated dysfunction in cardiomyocytes. *In vitro* studies have been conducted in mouse atrial HL-1 cardiomyocytes, which have phenotypic and morphological features very similar to that of adult cardiomyocytes (Claycomb et al., 1998). Oxidative stress has been induced in HL-1 cells by the mitochondrial stressor, Antimycin A and autophagy has been induced by mTOR inhibitor rapamycin. The in vitro study has been summarized in Chapter 2 of this dissertation. The in vivo study

comprises Chapter 3 and involves the use of the chemotherapeutic agent doxorubicin, a known mitochondrial stressor, to induce oxidative stress in the hearts of 26-month-old Fischer 344 x Brown Norway rats. CR and resveratrol, either alone or in combination, has been tested as interventions to induce autophagy in the hearts of these rats. The chapters provide novel results that further support the beneficial role of autophagy in the context of oxidatively stressed cardiomyocytes. Finally, the overall conclusions of the dissertation project as well as future directions will be discussed in Chapter 4. We believe that the research questions investigated in this study will likely provide answers to the role autophagy plays in alleviating oxidant-induced damage in cardiomyocytes.

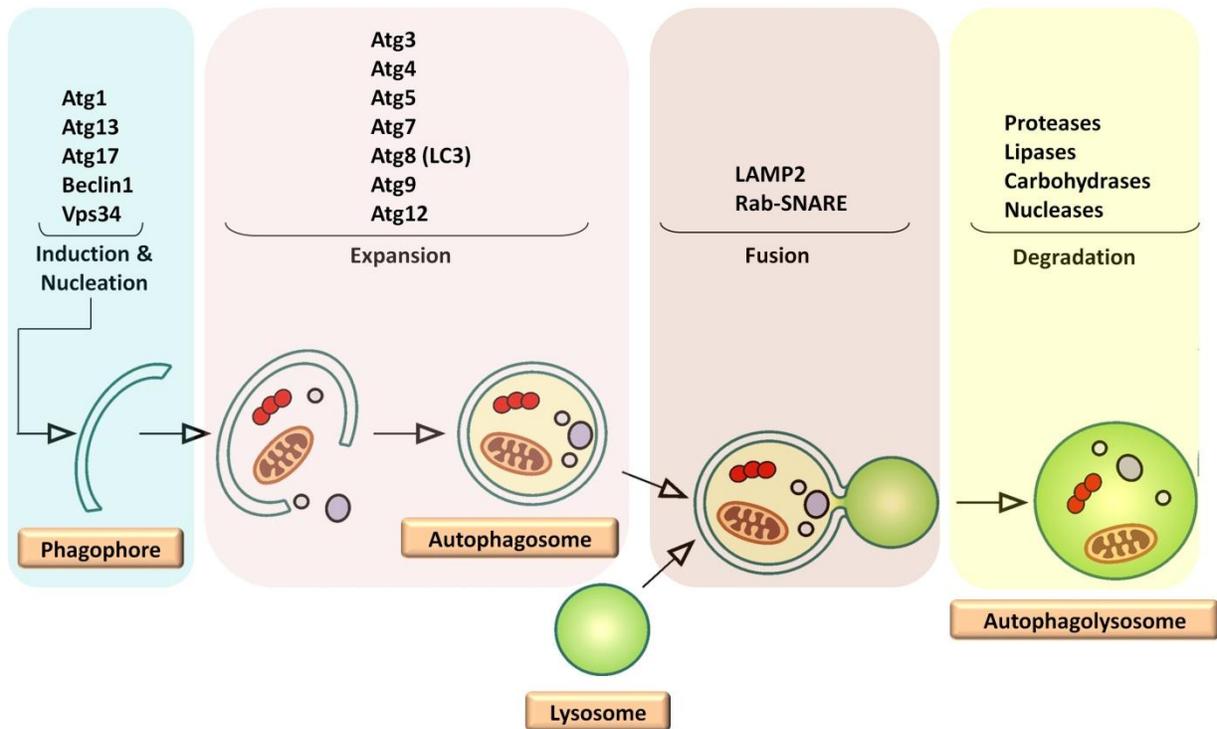
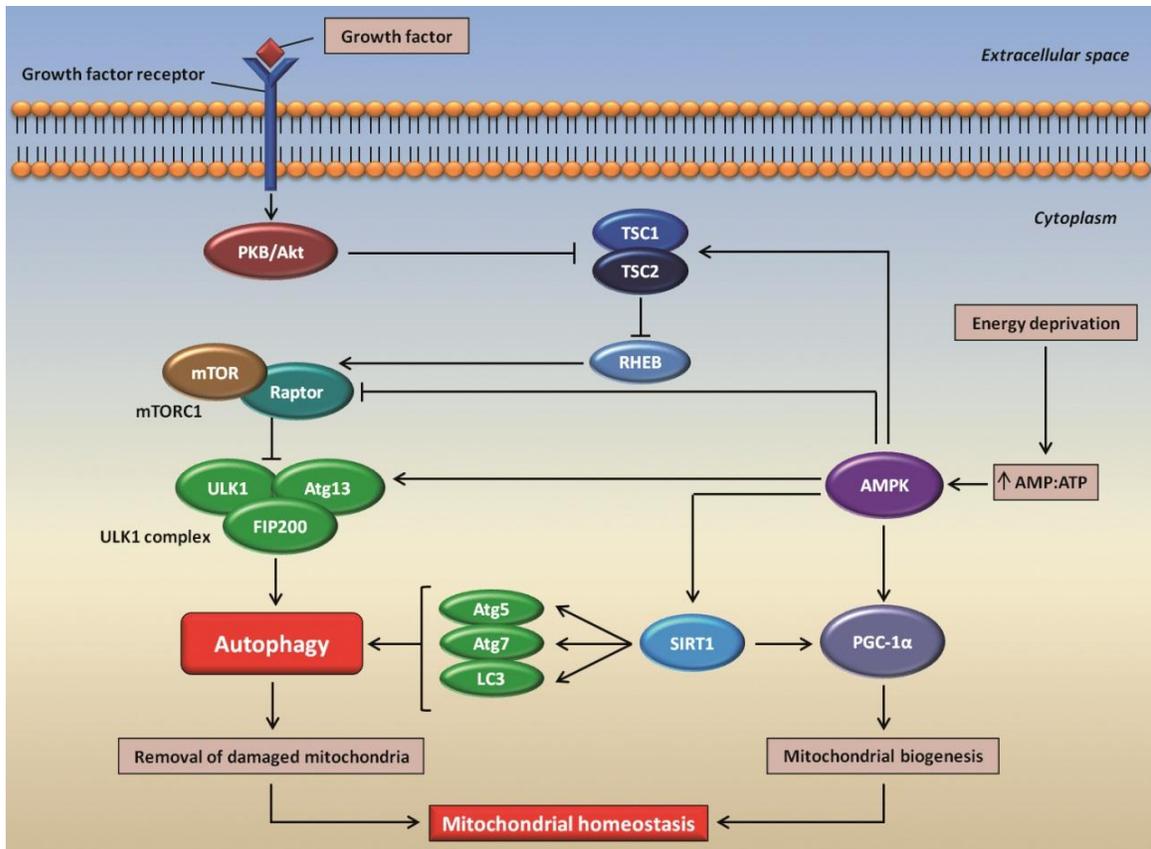


Figure. 1-1. Schematic representation of the distinct steps of the autophagic process. The process of autophagy can be dissected into several steps, comprising of *induction and nucleation*, *expansion*, *fusion* and *degradation*. Overall, autophagy begins with the formation of a double-layered isolation membrane (phagophore) around the molecules and/or organelles to be degraded. The phagophore grows in size and completely engulfs the cargo, forming an autophagosome. The autophagosome subsequently fuses with the lysosome evolving into an autophagolysosome wherein the cargo is digested. Some of the key molecular regulators controlling the autophagic process have also been shown. (Reproduced from Dutta et al., 2012).

Figure 1-2. Schematic overview of the molecular regulation of autophagy in the context of mitochondrial homeostasis. In the presence of nutrients and growth factors, the PKB/Akt pathway is activated, which blocks TSC1/TSC2 and relieving their inhibitory effect on RHEB. The latter, in turn, activates mTORC1. mTORC1 inhibits autophagy by blocking the ULK1-Atg13-FIP100 complex through inactivating phosphorylations on Atg1 and Atg13. Under conditions of energy depletion, the cellular AMP:ATP ratio increases and AMPK becomes activated, which in turn stimulates autophagy through multiple mechanisms. AMPK can phosphorylate and activate TSC1/TSC2, thereby relieving the mTORC1-mediated inhibition of autophagy. In addition, AMPK can phosphorylate Ulk1 at specific serine residues leading to the initiation of autophagy. AMPK can also inhibit the mTORC1 complex by phosphorylating Raptor (mTORC1 containing rapamycin-associated TOR protein), the binding partner required for mTORC1 activity. In addition, under nutrient starvation conditions, mTOR is inactivated and this removes the inactivating phosphorylation on Atg1 and Atg13, resulting in autophagy induction independent of AMPK. Once activated, autophagy serves multiple functions, including the clearance of damaged mitochondria. In addition AMPK induces mitochondrial biogenesis by activating PGC-1 α either directly or through SIRT1. SIRT1 can also deacetylate and activate several autophagy-related proteins, such as Atg5, Atg7 and LC3, resulting in enhanced autophagic activity. The autophagic removal of damaged mitochondria coupled with mitochondrial biogenesis is essential for the maintenance of mitochondrial homeostasis. (Reproduced from Dutta et al., 2012).

(see figure on next page)



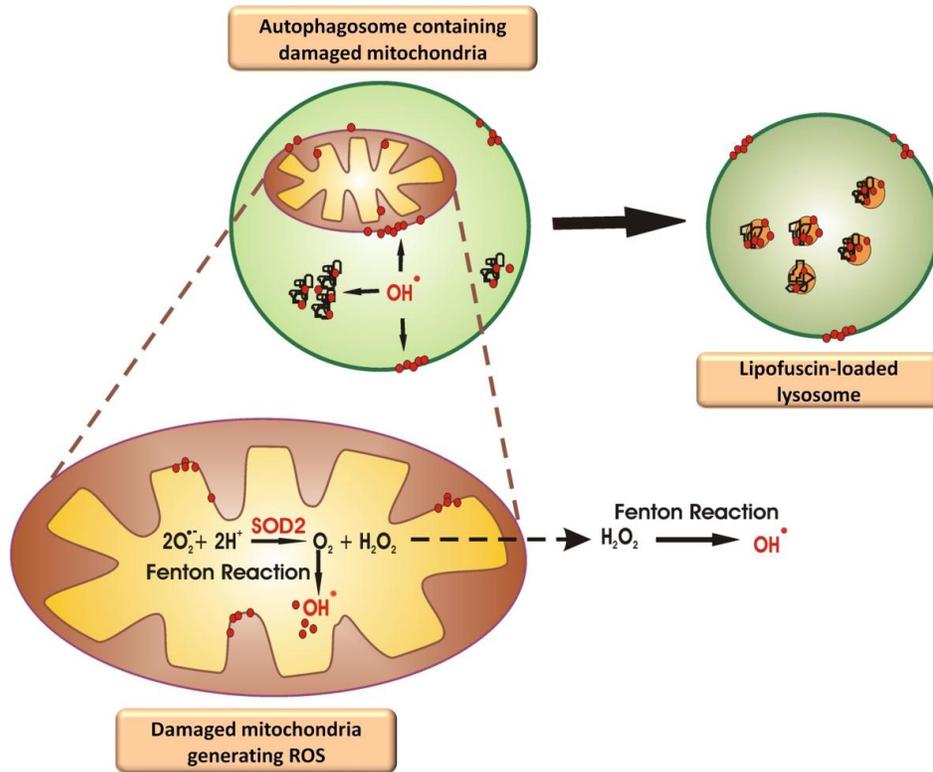


Figure 1-3. Schematic showing the contribution of mitochondria-derived ROS in the formation of lipofuscin in cells. Damaged mitochondria engulfed within an autolysosome generate hydroxyl radicals (OH^\bullet) via Fenton reactions. Hydrogen peroxide (H_2O_2) participating in these reactions is generated by the action of SOD2 on superoxide anion ($\text{O}_2^{\bullet-}$) produced by the electron transport chain. OH^\bullet causes crosslinking between intralysosomal proteins, lipids and/or proteins, forming an indigestible polymer called lipofuscin. (Reproduced from Dutta et al., 2012).

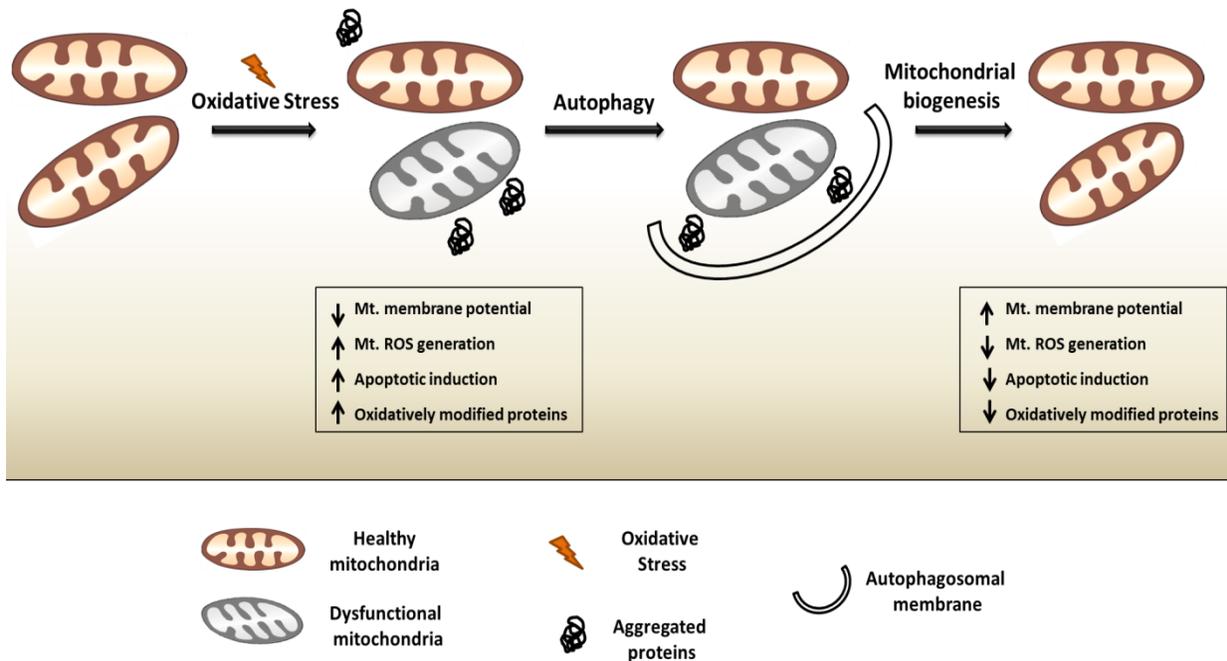


Figure 1-4. Schematic showing the beneficial effect of autophagy against oxidative stress-mediated dysfunction. Oxidative stress induces the accumulation of dysfunctional mitochondria and aggregated proteins in cells. Damaged mitochondria show decreased membrane potential, generate increased amounts of ROS and enhance apoptotic induction. Induction of autophagy mediates the removal of such dysfunctional mitochondria and oxidatively damaged proteins, which when coupled with functional biogenesis, helps preserve cellular homeostasis.

CHAPTER 2 UPREGULATED AUTOPHAGY PROTECTS CARDIOMYOCYTES FROM OXIDATIVE STRESS-INDUCED CYTOTOXICITY

Introduction

Progressive accumulation of mitochondrially-generated ROS has been proposed to be a major player in the pathogenesis of many chronic degenerative diseases, such as CVD (Balaban et al., 2005; Judge et al., 2005a). Experimental evidence indicates that in cardiac cells, damaged and dysfunctional mitochondria can produce up to 10 fold more hydrogen peroxide (H_2O_2) than intact organelles (Grivennikova et al., 2010). Oxidants, in low amounts, can function as essential signaling molecules (Finkel, 2011). In contrast, excessive ROS generation is detrimental, negatively affecting the function and integrity of not only the mitochondria, but also of proteins, lipids and other organelles in the cell, potentially leading to impaired cellular homeostasis and organ function (Judge & Leeuwenburgh, 2007; Lin & Beal, 2006). In fact, mitochondrial dysfunction and oxidative stress plays a major role in the pathogenesis of cardiac LVH as well as in the progression from compensated LVH to heart failure ((Abel & Doenst, 2011). Mitochondria-generated oxidative stress also plays a critical role in the pathogenesis of myocardial damage during ischemia/reperfusion, which eventually progresses to cardiac dysfunction (Lesnefsky et al., 2001). Further evidences for the involvement of mitochondrial derived oxidative stress in mammalian heart senescence is provided by the observation that overexpressing the anti-oxidant enzyme catalase in the mitochondria (mCAT) delays the development of cardiac pathology in mice (Dai et al., 2009b). The rate of H_2O_2 generation and mitochondrial oxidative damage are significantly decreased in the hearts of old mCAT mice compared with age-matched wild-type controls (Dai et al., 2009b).

Apart from the intra-mitochondrial proteolytic system selectively removing damaged proteins, the only known mechanism by which mitochondria can be cleared from a cell is through autophagy. Autophagy is a self-digestion process whereby the cell degrades long-lived proteins and organelles, generating amino acids and fatty acids that can be reutilized by the cell (Mizushima & Levine, 2010). Growing evidence suggests that autophagy could specifically target damaged and dysfunctional mitochondria for removal, which might otherwise lead to apoptotic induction (Tait & Green, 2010). Additionally, autophagy can also remove oxidatively modified and dysfunctional proteins as a bulk digestion process, thereby maintaining a “cleaner” cell and avoiding potential proteotoxicity arising from their unwanted accumulation (Cuervo et al., 2005). It is worth noting here that oxidative stress per se has been shown to induce autophagy, by specifically regulating the activity of Atg4 (Scherz-Shouval et al., 2007a). However, recent studies show that the type of oxidant generated and its site of origin are crucial factors in determining the extent and the result of such an autophagic induction (Dewaele et al., 2010).

Rapamycin is an immunosuppressant antibiotic widely used as an inducer of autophagy, acting through its inhibitory effect on mTOR (Ravikumar et al., 2004). In the presence of nutrients and growth factors, mTOR is active, hyperphosphorylating and inhibiting the ULK1 - Atg13 – FIP200 complex required for the induction of autophagy (Jung et al., 2009b). Rapamycin therefore primarily acts to remove the inhibitory effect of mTOR on autophagy. The cardioprotective properties of rapamycin have been demonstrated in animal models of ischemia / reperfusion as well as in cardiac hypertrophy (Khan et al., 2006; McMullen et al., 2004). In addition, rapamycin also

protects against neurodegeneration by mediating the removal of aggregated proteins through autophagy induction (Harada et al., 2008).

In this study, we investigated whether autophagy induction by rapamycin can be beneficial against mitochondrially-generated oxidative stress in a mouse cardiomyocyte HL-1 cell line, which has morphological and phenotypic characteristics very similar to adult cardiomyocytes (Claycomb et al., 1998). Mitochondrial stress has been induced by Antimycin A (AMA), a fungicide isolated from *Streptomyces kitazawensis* (Nakayama et al., 1956) and known to specifically inhibit complex III of ETC by binding to the Qi site of cytochrome *c* reductase (Potter & Reif, 1952). The binding results in the inhibition of flow of electrons through the ETC, generating O₂^{•-} in the mitochondria and inducing apoptosis (Pham et al., 2000).

Our study shows that in HL-1 cardiomyocytes, AMA mimics several pathologic features associated with mitochondrial dysfunction *in vivo*, i.e. increased generation of ROS, enhanced DNA oxidation and apoptotic induction. Autophagy induction by rapamycin decreases apoptotic induction, enhances the clearance of ubiquitinated proteins and importantly, improves mitochondrial function in AMA-treated cells. On the other hand, inhibition of autophagy blocks the beneficial effects of rapamycin. We suggest that rapamycin offers cytoprotection against oxidative stress by a combined approach of removing dysfunctional mitochondria as well as by degrading damaged, ubiquitinated proteins. We therefore propose autophagy induction as a potential therapeutic strategy against oxidative stress-mediated damage in cardiomyocytes.

Materials and Methods

Cell Culture Conditions

HL-1 mouse atrial cardiomyocytes were a kind gift of Dr. W Claycomb (Louisiana State University Health Science Center, LA). Cells were cultured in Claycomb Media (JRH Biosciences, KS) supplemented with 10% fetal bovine serum (Sigma, MO), 2 mM L-glutamine (Life Technologies, MD), 0.1 mM norepinephrine (Sigma, MO), 100 U/mL penicillin and 100 mg/mL streptomycin (Life Technologies, MD), on fibronectin-gelatin coated plates at 37°C in a humidified atmosphere, with 5% CO₂.

Chemical Reagents

AMA (Sigma, MO) was dissolved in ethanol at a concentration of 15 mM; Rapamycin (Calbiochem, CA) was made up in ethanol at 5 mM; BafA1 (Sigma, MO) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 25 µM and 3-MA (Sigma, MO) was dissolved in deionized water at 180 mM. A more complete list of all the reagents used in the study, their source and catalog numbers is given in table 2-1.

Flow Cytometric Determination of Mitochondrial O₂^{•-} Generation

O₂^{•-} generation in the mitochondria was analyzed using the fluorescent dye MitoSOX Red (Invitrogen, CA). It is specifically targeted to bioenergetically active mitochondria where it is rapidly and selectively oxidized by O₂^{•-} molecules. The oxidized product binds to the mitochondrial DNA and fluoresces red. (Mukhopadhyay et al., 2007) HL-1 cells were trypsinized and resuspended in fresh media at a density of 2x10⁶ cells/mL and MitoSOX Red was added to a final concentration of 3 µM. Cells were allowed to load MitoSOX Red for a period of 30 mins at 37°C, washed twice with Dulbecco's Phosphate Buffered Saline (DPBS) and the media replaced with fresh media. Cells were subsequently incubated with increasing concentrations of AMA or

vehicle control for 30 mins at 37°C, followed by Flow cytometric analysis of MitoSOX Red fluorescence using FACSCalibur flow cytometer (BD Biosciences, CA). MitoSOX Red was excited by laser at 488 nm and the data collected using forward scatter (FSC) and side scatter (SSC) and FL2 (575 +/- 12.5 nm) channels. Cell debris is represented by distinct low FSC and SSC and was gated out for analysis. The data shown represents mean MitoSOX Red fluorescence intensity of 10,000 HL-1 cells treated with the indicated concentrations of AMA.

Confocal Imaging of Mitochondrial O₂^{•-} Generation

Cells on No. 1.5 coverslip-glass bottom dishes (MatTek Corporation, MA) were incubated with 3 µM MitoSOX Red and 1 µg/mL Hoechst 33342 (Invitrogen, CA) for a period of 30 mins at 37°C, washed twice with DPBS and the media replaced with fresh media. Cells were subsequently treated with 50 µM AMA or vehicle control for 30 mins and imaged for MitoSOX Red fluorescence using Leica TCS SP2 Laser Scanning Confocal Microscope (Leica Microsystems GmbH, Heidelberg, Germany) using an excitation of 514 nm and emission of 560-600 nm. Hoechst 33342 was visualized using 405 nm excitation and 440-470 nm emission. The pictures were merged using Leica LCS software (Leica Microsystems GmbH, Heidelberg, Germany).

Flow Cytometric Determination of Mitochondrial Membrane Potential

$\Delta\psi_m$ was analyzed using the cationic fluorescent dye TMRM (Invitrogen, CA), which is specifically targeted to and accumulates in bioenergetically active mitochondria (Scaduto & Grotyohann, 1999). The dye is lost when the mitochondrial membrane potential ($\Delta\psi_m$) is lost. HL-1 cells were trypsinized and resuspended in fresh media at a density of 2×10^6 cells/mL, after which TMRM was added to a final concentration of 50 nM. Cells were allowed to load TMRM for a period of 30 mins at 37°C, washed twice

with DPBS and the media replaced with fresh media. Cells were subsequently incubated with increasing concentrations of AMA or vehicle control for 2h at 37°C, followed by Flow cytometric analysis of TMRM fluorescence using the same excitation-emission and gating protocol as that of MitoSOX Red.

Confocal Imaging of Mitochondrial Membrane Potential

Cells were incubated with 50 nM TMRM and 1 µg/mL Hoechst 33342 at 37°C for a period of 30 mins, washed twice with DPBS and the media replaced with fresh media. Cells were subsequently treated with 50 µM AMA or vehicle control for 2h and imaged for TMRM fluorescence using Leica TCS SP2 Laser Scanning Confocal Microscope (Leica Microsystems GmbH, Heidelberg, Germany) with an excitation of 543 nm and emission of 560-600 nm. The pictures were merged using Leica LCS software (Leica Microsystems GmbH, Heidelberg, Germany).

Determination of Cell Viability

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, MO) and Trypan Blue dye exclusion assay (Sigma, MO). To determine cell viability by MTT assay, cells in 24-well plates were incubated with 0.5 mg/mL MTT for a period of 30 mins at 37°C. The purple formazan crystals formed were dissolved in DMSO at 37°C for 10 mins and the absorbance of the resulting solution measured at 540 nm using Biotek Synergy plate reader (Biotek, VT). The background absorbance was subtracted at 630 nm. The values represented are normalized to control vehicle-treated group. To determine cell viability by Trypan Blue assay, cells were trypsinized and resuspended in fresh media containing equal volume of 0.4% Trypan Blue (Sigma, MO) for 5 mins and the number of live (bright) or dead (dark) cells counted using a hemocytometer.

Determination of DNA / RNA Oxidation

8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dGuo; measure of DNA oxidation) and 8-oxo-7,8-dihydroguanosine (8-oxo-Guo; measure of RNA oxidation) concentrations were measured in cells using HPLC coupled to electrochemical detection (HPLC-ECD) analysis (ESA Coulochem III electrochemical detector, MA) according to protocol described before (Hofer et al., 2006). Briefly, cells were treated with AMA or vehicle control for 2h and 24h later, they were trypsinized and subsequently homogenized in 3 M guanidium thiocyanate lysis buffer (GTC, 0.2% w/v N-lauroylsarcosinate, 20 mM Tris, pH 7.5) in the presence of the metal chelator deferoxamine mesylate (DFOM; Sigma, MO) to prevent further oxidation. Cellular DNA / RNA was isolated using phenol-chloroform method and precipitated in isopropanol. The nucleic acid pellet was subsequently washed with ethanol, air-dried and dissolved in 10 mM DFOM followed by hydrolysis in 10 μ L Nuclease P1 (stock of 0.4 U/ μ L in 300 mM sodium acetate, 0.2 mM ZnCl₂, pH 5.3; MP Biomedicals, OH) and 5 μ L alkaline phosphatase (1 U/ μ L; Sigma, MO) for a period of 60 mins at 50°C. The hydrolysate was filtered to remove enzymes and subsequently loaded for HPLC-ECD analysis. For quantification, HPLC peaks were compared to calibration curves of 8-oxo-dGuo and dGuo standards (Sigma, MO). The amount of 8-oxo-Guo and 8-oxo-dGuo present was normalized to the total amount of non-oxidized DNA and RNA respectively.

Induction and Assessment of Autophagy

Autophagic stimulation converts the cytosolic and unconjugated form of LC3 (LC3-I) to the carboxy-terminal cleaved, phosphatidylethanolamine (PE) conjugated form (LC3-II) and this causes a change in the molecular weight of the protein, allowing both the forms to be resolved in an SDS-PAGE gel. The LC3-II/LC3-I ratio is therefore

indicative of the amount of ongoing autophagy. However, an increase in the LC3-II/LC3-I ratio could be due to increased autophagic stimulation or due to a decreased clearance of autophagic vacuoles in the lysosomes. Hence all autophagic responses have been monitored in the absence or presence of the lysosomal inhibitor BafA1, to determine *steady state* and *cumulative autophagy* (*a.k.a* autophagic flux), respectively. To determine the optimum concentration of rapamycin for inducing autophagy in HL-1 cardiomyocytes, cells were treated with increasing concentrations of the drug for a period of 16h in the presence or absence of 75 nM BafA1, added during the final 4h of incubation. For subsequent experiments involving rapamycin, a concentration of 1 μ M was used.

Epifluorescence Imaging and Quantification of Autophagy

For assessment of autophagic activity using epifluorescence imaging, cells plated on coverslips in 24-well plates were transfected with GFP-LC3 plasmid (kind gift of Dr T. Yoshimori, National Institute of Genetics, Japan) (Kabeya et al., 2000) using Lipofectamine 2000 (Invitrogen, CA) following manufacturer's instructions. To prevent the potential problem of GFP-LC3 aggregate formation (Kuma et al., 2007), transfection was optimized with a low concentration of GFP-LC3 plasmid (0.8 μ g for each well of a 24 well plate). Twenty-four hours after transfection, cells were incubated with 1 μ M rapamycin for 16h, in the absence or presence of 75 nM BafA1 added during the final 4h of incubation. Cells were subsequently by fixed in 4% paraformaldehyde and mounted on slides with DAPI (Vector Laboratories, CA). Epifluorescence images were taken using Leica DM IRBE epifluorescence microscope (Leica Microsystems, Rueil-Malmaison, France) and images were processed using ImageJ software (NIH, MD). Conversion of diffuse cytosolic GFP-LC3-I to punctate autophagic membrane-

associated GFP-LC3-II was monitored and considered indicative of autophagic activity in cells. Specifically, the number of puncta in each cell was counted and cells with 30 or more puncta were considered to have induced autophagy.

Confocal Imaging

Confocal microscopy was used for live-cell imaging of autophagy and mitophagy in AMA and AMA plus rapamycin-treated cells. Cells were plated on 0.17 mm thick glass-bottom Fluorodishes (World Precision Instruments, FL) and were transfected with GFP-LC3 plasmid using Lipofectamine 2000 (Invitrogen, CA) following manufacturer's instructions. Twenty-four hours after transfection, cells were incubated with 1 μ M rapamycin or vehicle control for 16h. Cells were subsequently treated with 50 μ M AMA and the autophagic vacuoles visualized using Zeiss LSM 510 Meta laser scanning microscope (Zeiss, Germany). For visualization of mitophagy, cells were pre-loaded with 50 nM TMRM for 30 mins at 37°C before the addition of 50 μ M AMA. GFP fluorescence was excited at 488 nm and emission was collected through a 500 - 530 nm band-pass filter. TMRM was excited at 545 nm and emission collected through a 565 – 619 nm band pass filter. Confocal images were processed using LSM Image Browser software (Zeiss, Germany).

Western Blotting

Cells were lysed using RIPA buffer (Sigma, MO) and protein concentration determined using Dc assay (BioRad, CA) (Bradford, 1976). Equal amounts of proteins were then loaded in pre-cast Tris-HCl polyacrylamide gels (BioRad, CA). After electrophoretic separation, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (BioRad, CA) and subsequently blocked for 1h in Starting Block (Thermo Scientific, NJ), followed by overnight incubation with primary antibodies. Membranes

were subsequently washed three times with Tris buffered saline with 0.1% Tween 20 (TBST) and incubated with the appropriate secondary antibodies for 1h. Membranes were washed again with TBST and chemiluminescent signals developed using ECL Plus reagent (Amersham Biosciences, Buckinghamshire, UK). The signals were captured using ChemiDoc XRS System (BioRad, CA) and digital images analyzed using ImageLab software (BioRad, CA). A list of primary antibodies used in the study, their source and catalog numbers is given in Table 2-2.

For detection of ubiquitinated proteins, cells were lysed and detergent soluble and insoluble fractions separated by centrifugation at 8000g. The resulting pellets (insoluble fraction) were resuspended in 2X SDS buffer and heated at 100°C for 10 mins. Equal volumes of sample lysates were then loaded in each lane. The supernatant (soluble fraction) was processed as mentioned above.

Assessment of Cellular Respiration

Cells were pre-treated with 1 μ M rapamycin or vehicle control for 16h, followed by incubation with 50 μ M AMA or vehicle control for an additional 24h, in the presence of rapamycin. Cells were subsequently trypsinized and resuspended in complete Claycomb media. The O₂ flux, as a measure of routine respiration was subsequently measured in unpermeabilized cells using OROBOROS Oxygraph-2K (Oroboros Instruments GmbH, Innsbruck, Austria) at 37°C in a constantly stirred chamber. The rates of consumption are expressed as pmol / sec / 10⁶ cells.

Statistical Analysis

All treatments were done in three or more independent experiments. All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, Inc. San

Diego, CA). For statistical analysis, Student's *t*-test and one-way ANOVA were performed, wherever applicable. Statistical significance was set to $p < 0.05$ and the data are represented as mean \pm SE.

Results

AMA Increases Mitochondrial $O_2^{\cdot-}$ Generation and Decreases Mitochondrial Membrane Potential ($\Delta\psi_m$)

First, we established the concentration of AMA needed to increase ROS generation in the mitochondria. Cells pre-labeled with MitoSOX Red, highly selective for the detection of $O_2^{\cdot-}$ in the mitochondria (Mukhopadhyay et al., 2007), were treated with increasing concentrations of AMA or vehicle control and the fluorescence intensity analyzed using flow cytometry. In contrast to vehicle-treated cells which showed minimal MitoSOX Red fluorescence, treatment with AMA resulted in a dose-dependent increase in fluorescence intensity, with 50 μ M being the lowest concentration required to reach statistical significance (Fig. 2-1A; $p < 0.05$). To confirm flow cytometry results, we performed confocal imaging on cells pre-labeled with MitoSOX Red and treated with 50 μ M AMA. In contrast to vehicle-treated cells which showed minimal fluorescence, treatment with 50 μ M AMA resulted in a strong MitoSOX Red fluorescence originating from the mitochondria (Fig. 2-1B).

Next, we determined the effects of AMA on $\Delta\psi_m$. These studies were done in cells pre-labeled with Tetramethyl Rhodamine Methyl Ester (TMRM), a cationic, fluorogenic dye which specifically migrates to bioenergetically active mitochondria and fluoresces red (Scaduto & Grotyohann, 1999). TMRM-loaded cells were treated with increasing concentrations of AMA or vehicle control and the fluorescence intensity analyzed using flow cytometry. Vehicle-treated cells showed a strong fluorescence, indicative of normal

membrane potential, whereas AMA treatment resulted in a dose dependent decrease in TMRM fluorescence (Fig. 2-1C). We observed that 50 μ M is the lowest concentration of AMA required to reach statistically significant decreases in $\Delta\psi_m$ ($p < 0.05$). Confocal imaging further confirmed that 50 μ M AMA resulted in a drastic decrease in TMRM fluorescence, compared to vehicle-treated cells (Fig. 2-1D).

AMA Treatment Induces Nuclear DNA Oxidation and Decreases Cell Viability

We next wanted to investigate whether AMA-induced ROS can leak out of the mitochondria and have damaging effects on extra-mitochondrial components. To test this, we measured the levels of 8-oxo-dGuo and 8-oxoGuo as an indicator of DNA and RNA oxidation, respectively (Hofer et al., 2006). AMA significantly increased the level of DNA oxidation in HL-1 cells at a concentration of 50 μ M (Fig. 2-1E, $p < 0.05$). RNA oxidation tended to increase with all concentrations, but changes were not statistically significant (Fig. 2-1F; $p < 0.10$). Finally, the survival of HL-1 cells treated with increasing concentrations of AMA, as determined by MTT assay at different time points, showed a dose- and time-dependent decrease in viability (Fig. 2-1G). It is worth noting here that in addition to being used as a viability marker, the MTT assay is also indicative of mitochondrial activity, as most of the dehydrogenases required for the reduction of MTT reside in the mitochondria.

Treatment with AMA does not Induce Autophagy in HL-1 Cells

Since oxidative stress has been shown to be an inducer of autophagy, we wanted to determine whether treatment with AMA can lead to autophagy induction in HL-1 cells. Cells were treated with increasing concentrations of AMA for either 4h or 24h in the absence or presence of Bafilomycin A1 (BafA1), added during the final 4h or 2h of incubation, respectively. At the 24h time point, cells were incubated with BafA1 for a

shorter period of time to avoid additional toxicity in stressed HL-1 cells. Immunoblot analysis showed no significant increases in the LC3-II/LC3-I ratio at either time points with any of the concentrations of AMA tested (Fig. 2-2A, C). Epifluorescence analysis of GFP-LC3 puncta in AMA treated cells further confirmed no induction of autophagy by AMA ($p > 0.05$, Fig. 2-2B). In addition, we assessed the levels of other autophagy proteins Atg5-Atg12, Beclin-1 and Atg7 and detected no difference in their expression levels in AMA treated cells (Fig. 2-2D). We ruled out the possibility that this inability to induce autophagy is due to an apparently high oxidative burden generated by the concentrations of AMA used, as lower concentrations (50 nM) had no effect on the LC3-II/LC3-I ratio as well (data not shown).

Treatment with Rapamycin Induces Autophagy in HL-1 Cells

Incubation of HL-1 cardiomyocytes with rapamycin caused an inhibition of mTOR signaling, as shown by the disappearance of phospho-S6, one of the downstream targets of mTOR kinase (Fig. 2-3A). Additionally, rapamycin caused a significant increase in cumulative LC3-II/LC3-I ratio at a concentration of 1 μ M ($p < 0.01$), with lower concentrations not reaching statistical significance (Fig. 2-3B). As a further confirmation of our immunoblotting data, we performed epifluorescence imaging on GFP-LC3 expressing cells to determine steady state and cumulative autophagy (Fig. 2-3C). For these experiments, cells were incubated with 1 μ M rapamycin or vehicle control for 16h, in the absence or presence of BafA1 present during the final 4h of incubation. Quantification of cumulative GFP-LC3 puncta further confirmed induction of autophagy by rapamycin (Fig. 2-3D) ($p < 0.001$). We further observed that treatment with 1 μ M rapamycin decreased mitochondrial content, as observed by the disappearance of the mitochondrial outer membrane marker VDAC (Fig. 2-3E).

Additionally, we confirmed that rapamycin can induce autophagy in the presence of AMA, both by immunoblotting (Fig. 2-4A) and confocal imaging in GFP-LC3 transfected cells (Fig. 2-4B). Furthermore, rapamycin also led to the degradation of mitochondria in AMA-treated cells (Fig. 2-4C). Importantly, the reported half-life of rapamycin is 63h (Backman et al., 2002), confirming its chemical stability during the experimental period.

Rapamycin Plays a Protective Role against AMA-Induced Toxicity

We next investigated whether rapamycin can mediate a protective effect against AMA-induced toxicity. For these experiments, cells were pre-treated with rapamycin for 16h, followed by addition of AMA in fresh media in the presence of rapamycin. Viability analysis, both by MTT (Fig. 2-5A) and Trypan Blue exclusion assays (Fig. 2-5B) revealed a drastic decrease ($p < 0.01$) in cell viability with AMA treatment, which was significantly attenuated by treatment with rapamycin ($p < 0.05$). MTT assay revealed a slight decrease in cell viability due to rapamycin treatment alone (Fig. 2-5A), but the changes were not statistically significant and we believe this is to be due to the anti-proliferative effect of the mTOR inhibitor per se, which led to a slightly decreased cell number (as opposed to cell death) in comparison to vehicle-treated cells. This is further supported by the observation that there was no difference in the number of live cells in control and rapamycin-treated cells when viability was assessed by Trypan Blue dye exclusion assay (Fig. 2-5B).

As compared to control or rapamycin-treated cells, AMA induced both caspase-3 and PARP-1 cleavage in HL-1 cells, and rapamycin treatment significantly attenuated such proteolytic processing (Fig. 2-5C). Additionally, when observed by phase contrast microscopy, AMA-treated cells showed intense vacuolization in the cytoplasm, a phenomenon often observed with injury, toxicity and cell death (Henics & Wheatley,

1999) Treatment with rapamycin drastically decreased the appearance of such vacuoles (Fig. 2-5D).

Rapamycin Prevents AMA-Induced Accumulation of Ubiquitinated Proteins

The accumulation of high molecular weight ubiquitinated proteins has been observed under conditions of increased oxidative stress and can originate from an inefficient clearance of damaged proteins through the proteasomal system (Dudek et al., 2005; Shang & Taylor, 2011). Under such conditions, autophagy induction can mediate their removal, thereby averting potential proteotoxicity. We found that treatment with AMA induced the accumulation of ubiquitinated proteins in the detergent-insoluble fraction and treatment with rapamycin drastically prevented such an accumulation (Fig. 2-6). Interestingly, in comparison to control, we observed a decreased accumulation of ubiquitinated proteins in cells treated with rapamycin alone. This could be attributed to the autophagic clearance of basal levels of ubiquitinated proteins by rapamycin.

Rapamycin Protects against AMA-Induced Mitochondrial Dysfunction

We further wanted to investigate whether rapamycin provides any beneficial effect on the mitochondrial population in AMA-treated cells. In this respect, assessing cellular respiration in non-permeabilized cells using polarographic oxygen sensors has been shown to be an accurate measure of mitochondrial function (Gnaiger, 2008). We found that AMA-treated cells significantly decreased routine respiration even 24h after treatment ($p < 0.01$). This decline in respiration was ameliorated by rapamycin treatment ($p < 0.05$) (Fig. 2-7A).

We also measured $\Delta\psi_m$ as an additional indicator of mitochondrial function. In comparison to control or rapamycin-treated cells AMA-treated cells had mitochondria with little or no $\Delta\psi_m$ even 24h after treatment (arrows, Fig. 2-7B). The few cells that

maintained $\Delta\psi_m$ showed drastically altered mitochondrial morphology, most of them being highly swollen and drastically bigger in size (arrowheads, Fig. 2-7B inset).

Incubation with rapamycin restored $\Delta\psi_m$ in AMA-treated cells, as well as maintained normal mitochondrial morphology (Fig. 2-7B).

Inhibition of Autophagy Blocks the Cytoprotective Effect of Rapamycin against AMA Toxicity

We next determined whether the cytoprotective effect of rapamycin against AMA toxicity is specifically mediated through autophagy. For this, autophagy was inhibited by co-incubating cells with 3-Methyladenine (3-MA), a widely used inhibitor of autophagy (Seglen & Gordon, 1982; Stroikin et al., 2004). LC3-II/LC3-I ratios confirmed that 3-MA inhibited rapamycin-induced autophagy (Fig. 2-8A). We subsequently performed MTT assay to determine the viability of AMA-treated cells, co-incubated with rapamycin alone or with rapamycin in the presence of 3-MA (Fig. 2-8B). The MTT assay revealed that 3-MA attenuated the cytoprotective effects of rapamycin against AMA toxicity (Fig. 2-8B). In addition, 3-MA inhibited rapamycin induced attenuation of PARP-1 caspase-3 cleavage in AMA-treated cells (Fig. 2-8C).

Discussion

Mitochondria from heart failure models generate larger amounts of $O_2^{\cdot-}$ in the presence of NADH compared with normal mitochondria (Ide et al., 1999). Such oxidative stress can activate signaling pathways involved in myocardial remodeling, affecting cardiomyocyte structure and function (Ide et al., 1999). Our study shows that the mitochondrial stressor AMA can mimic several features observed under mitochondrial dysfunction-associated pathological conditions. For instance, AMA treatment generated $O_2^{\cdot-}$ in the mitochondria and decreased $\Delta\psi_m$ in HL-1

cardiomyocytes. A decreased $\Delta\psi_m$ is indicative of defective oxidative phosphorylation and a lower capacity of generating ATP (Huttemann et al., 2008); it is therefore used as a marker of cellular and mitochondrial viability (Vayssier-Taussat et al., 2002). Importantly, although AMA enhanced $O_2^{\cdot-}$ generation in the mitochondria, it is worthwhile to note that under physiologically normal conditions, to counteract oxidative stress, the mitochondria and the cytoplasm are equipped with detoxifying enzymes and non-enzymatic anti-oxidants (Andreyev et al., 2005b). Therefore, oxidants generated by the mitochondria could potentially be detoxified by cellular anti-oxidant defense systems, preventing damage to cytoplasmic components (Andreyev et al., 2005b). However, under conditions of stress and disease pathology, damaged mitochondria can generate excessive amounts of ROS, overwhelm the defenses, and oxidatively modify extra-mitochondrial components, such as the DNA and RNA. The DNA base oxidation product, 8-oxo-dGuo, is potentially mutagenic (Cheng et al., 1992; Kuchino et al., 1987) and is widely used for the estimation of oxidative stress in tissues and urine (Gedik & Collins, 2005; Lin et al., 2004). We confirmed that ROS generated by AMA can increase 8-oxo-dGuo levels in HL-1 cardiomyocytes.

As regards the mitochondria, we observed that most cells treated with AMA had little or no $\Delta\psi_m$ and the mitochondria were highly swollen. Such aberrant mitochondria, often also characterized by loss of cristae and matrix derangements, has been observed through ultrastructural analysis of the myocardium of aged rodents (Sachs et al., 1977a) and in other models of mitochondrial injury (Bova et al., 2005), and is considered a marker of mitochondrial toxicity (Kaufmann et al., 2006; Masubuchi et al., 2006). Furthermore, AMA decreased cell viability and activated apoptotic markers, as

well as inhibited cellular respiration in HL-1 cardiomyocytes, further adding to cytotoxicity (Fig. 2-9).

Some findings suggest that mitochondrial stress and ROS per se can act as inducers of autophagy (Chen et al., 2009; Kiffin et al., 2006; Scherz-Shouval et al., 2007a). Other studies have shown that mitochondrial dysfunction and depolarization might not be sufficient to induce autophagy (Mendl et al., 2011). In our studies we could not detect any induction of autophagy by treatment with AMA alone, but rapamycin in the presence of AMA was capable of inducing autophagy. It is possible that additional triggers might be necessary but missing in AMA induced toxicity (Mendl et al., 2011). Furthermore, it has been proposed that the inhibition of ETC complex III can actually have an inhibitory effect on autophagy induction, although the molecular mechanisms are as of yet, unknown (Ma et al., 2011). Nevertheless, stimulated autophagy under oxidative stress conditions could be hypothesized to potentially remove oxidized macromolecules.

Since oxidative stress is postulated to be a major player in the pathogenesis of cardiovascular diseases, one might argue that the administration of antioxidants will offer protection against oxidant-mediated damage. However, clinical studies have been controversial, with limited or no cardiac benefit reported with chronic administration of anti-oxidants (Bjelakovic et al., 2007; Levonen et al., 2008; Tinkel et al.). Therefore approaches to remove oxidatively modified macromolecules through autophagy induction represent an appealing alternative. In our study, we observed that rapamycin induced autophagy in HL-1 cardiomyocytes (Fig. 2-3). Rapamycin-stimulated autophagy had a beneficial effect on AMA-treated cells, as assessed by the attenuation of

caspase-3 and PARP-1 cleavage, as well as by an improved survival of HL-1 cardiomyocytes (Figs. 2-5 and 2-9). Rapamycin treatment also inhibited the decrease in $\Delta\psi_m$ and prevented the appearance of swollen morphology of the mitochondria (Figs. 2-7 and 2-9). Additionally, respiration analysis in non-permeabilized HL-1 cells showed that autophagy induction by rapamycin attenuated the decline in cellular respiration induced by AMA (Figs. 2-7 and 2-9). We suggest that these protective effects of rapamycin could be partly due to the result of autophagy-mediated removal of dysfunctional mitochondria, which if not removed, can produce greater amounts of ROS and / or induce apoptosis through the release of pro-apoptotic factors usually sequestered in the intermembrane space (Tait & Green, 2010). Supporting this claim, we have observed rapamycin-induced degradation of mitochondria (mitophagy) in AMA-treated cells (Fig. 2-4C). The encapsulation and degradation of dysfunctional mitochondria by rapamycin-induced autophagy has also been reported elsewhere (Pan et al., 2009; Ravikumar et al., 2006). Additionally, mitochondrial clearance by rapamycin has been confirmed in our study (Fig. 2-3E). The clearance of dysfunctional mitochondria is further supported by our imaging analysis, where an accumulation of dysfunctional (lacking $\Delta\psi_m$) and swollen mitochondria was seen only in AMA-treated cells; such dysfunctional mitochondria could not be detected in cells additionally treated with rapamycin. Finally, we observed that respiration was inhibited to the same extent in AMA and AMA plus rapamycin treated cells 2h after treatment (data not shown). However, after 24 hours, respiration was significantly increased in AMA-treated cells additionally exposed to rapamycin, suggesting that some of the dysfunctional mitochondria might have been removed by autophagy during this time period. In this

context, fairly modest reductions in mitochondrial content can have significant effects in improving cell viability, because of a major positive-feedback component involved in apoptotic signaling by damaged mitochondria. Finally, in order to establish that the protective effects of rapamycin against AMA toxicity is autophagy specific, we used 3-MA (Seglen & Gordon, 1982); (Stroikin et al., 2004), known to inhibit Class III PI3K, required during the initial steps of autophagy (Petiot et al., 2000). We observed that 3-MA not only inhibited rapamycin induced autophagy, but also attenuated the beneficial effects of rapamycin against AMA toxicity.

Consistent with our observations, autophagy has been shown to play a protective role against anoxia-regeneration in isolated cardiomyocytes (Dosenko et al., 2006), as well as required for the pre-conditioning effect of adenosine (Cohen & Downey, 2008). On the other hand, inhibition of autophagy through cardiac specific deletion of the autophagy protein Atg5 results in mitochondrial misalignment and cardiac hypertrophy in response to pressure overload induced by transverse aortic constriction. This eventually progresses into left ventricular hypertrophy, contractile dysfunction and cardiac dilatation (Nakai et al., 2007a). Inhibition of the autophagic clearance pathway has also been implicated in the pathogenesis of Danon disease, which is characterized by cardiomyopathy and heart failure (Kitsis et al., 2007).

Similar to mitochondria, an increase in the 'dwell time' of oxidatively modified cytoplasmic proteins enhances their probability of being post-translationally modified, which could further increase cellular toxicity (Gershon & Gershon, 1970; Miquel et al., 1974). Damaged cellular components are marked by ubiquitination, to be subsequently removed by the ubiquitin-proteasomal system (UPS) (Shang & Taylor, 2011). Growing

evidence suggest that under conditions of increased oxidative burden, accumulation of high molecular weight ubiquitinated proteins is frequently observed, owing to their inefficient removal through UPS (Dudek et al., 2005; Iwai et al., 1998; Shang & Taylor, 2011). Autophagy induction under these circumstances has been shown to mediate the removal of such accumulated proteins. In our studies, rapamycin-induced autophagy attenuated the accumulation of ubiquitinated proteins that resulted from AMA-induced exposure. Such an accumulation was specifically detected in the insoluble fraction, suggesting that they might be aggregated in nature. Notably, our observation is consistent with studies showing that autophagy induction alleviates toxicity in cells expressing mutant aggregate prone proteins such as α -synuclein (Ravikumar et al., 2004) and huntingtin (Ravikumar et al., 2004; Webb et al., 2003).

In summary, it is important to note that cardiomyocytes are terminally differentiated; therefore any damages accumulated during its lifespan of several decades cannot be diluted by cell proliferation. Furthermore, the heart is highly metabolic and hence challenged with a considerable amount of oxidative stress during its lifetime. Hence, the maintenance of a functional pool of mitochondria, coupled with the removal of aggregated and potentially harmful cytoplasmic proteins are vital for the preservation of cardiomyocyte homeostasis. Our study conclusively proves that autophagy induction by rapamycin can mediate the removal of dysfunctional mitochondria as well as aggregated, ubiquitinated proteins in cardiomyocytes, leading to a protective effect of autophagy induction against oxidative stress (Fig. 2-9). We therefore propose rapamycin as a potential therapeutic strategy against cardiovascular disorders associated with chronic generation of oxidation stress.

Figure 2-1. Treatment with AMA leads to enhanced oxidative stress and cytotoxicity in HL-1 cardiomyocytes. (A) HL-1 cells were trypsinized and resuspended in fresh media followed by staining with 3 μ M MitoSOX Red. Cells were subsequently incubated with increasing concentrations of AMA or vehicle control for 30 mins followed by flow cytometric analysis of MitoSOX Red fluorescence. (B) Cells were incubated with 1 μ g/uL Hoechst 33342 (blue) and 3 μ M MitoSOX Red and subsequently treated with 50 μ M AMA or vehicle control for 30 mins, followed by confocal imaging. (C) Cells were trypsinized and resuspended in fresh media followed by staining with 50 nM TMRM (red) and were subsequently incubated with increasing concentrations of AMA or vehicle control for 2h followed by flow cytometric analysis of TMRM fluorescence. (D) Cells were incubated with 1 μ g/ul Hoechst 33342 and 50 nM TMRM and subsequently treated with 50 μ M AMA or vehicle control for 2h, followed by confocal imaging. (E) Cells were incubated with increasing concentrations of AMA for 2h and 24h later, trypsinized and processed for HPLC analysis of DNA (E) and RNA (F) oxidation. Data has been normalized to vehicle-treated control. (G) Cells were incubated with increasing concentrations of AMA and cell viability determined using MTT assay after the indicated time points. Data has been normalized to vehicle-treated control. *p < 0.05; **p < 0.01, ***p < 0.001 vs. control. Data represents mean \pm SE and is derived from three independent experiments.

(see figure on next page)

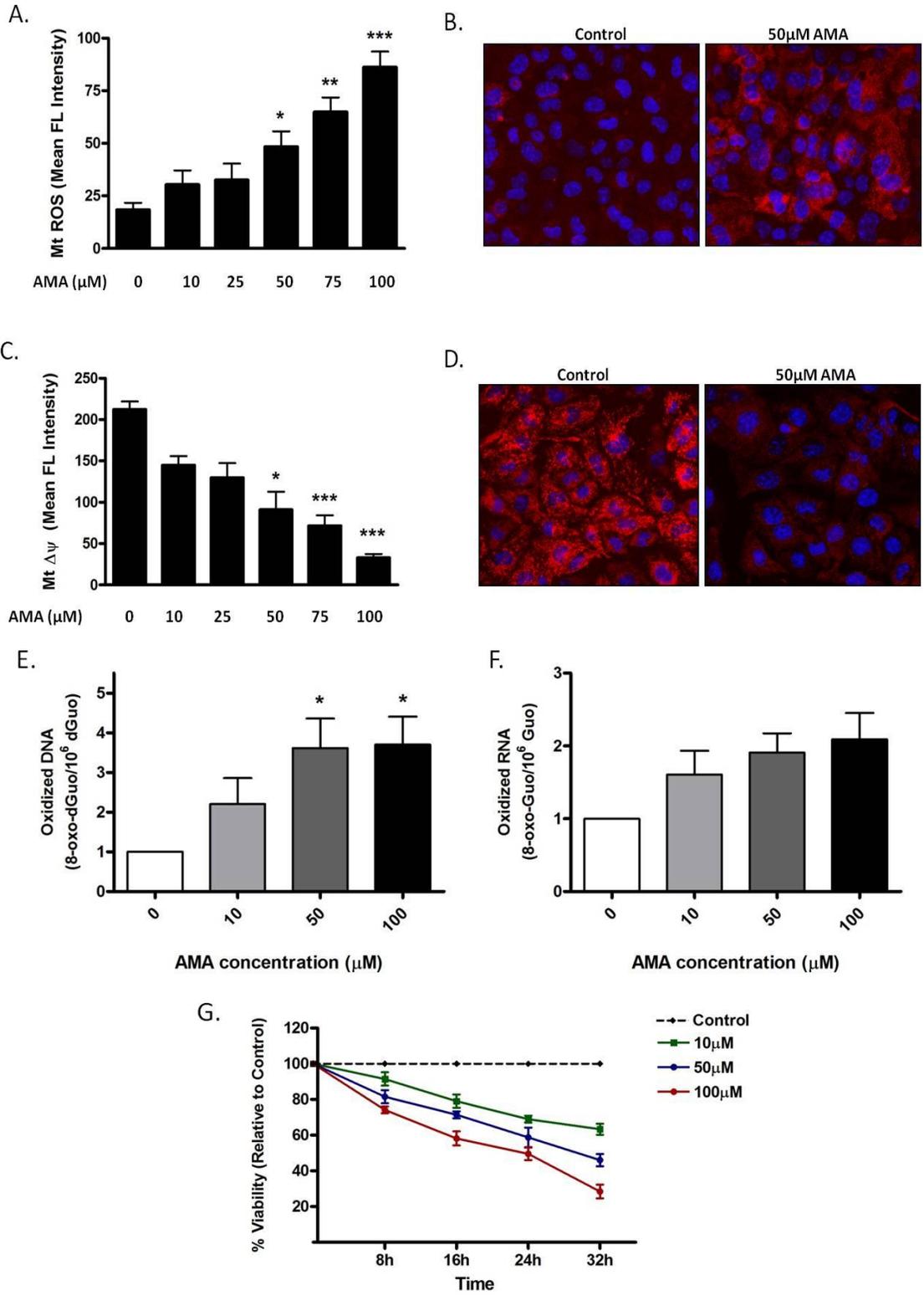
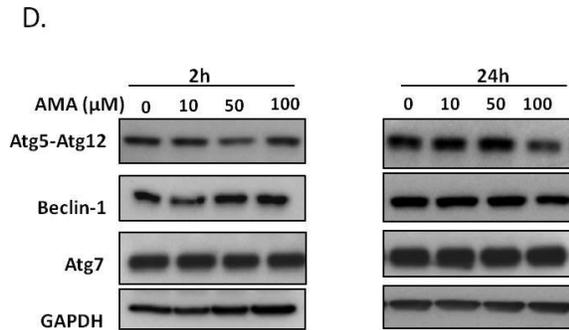
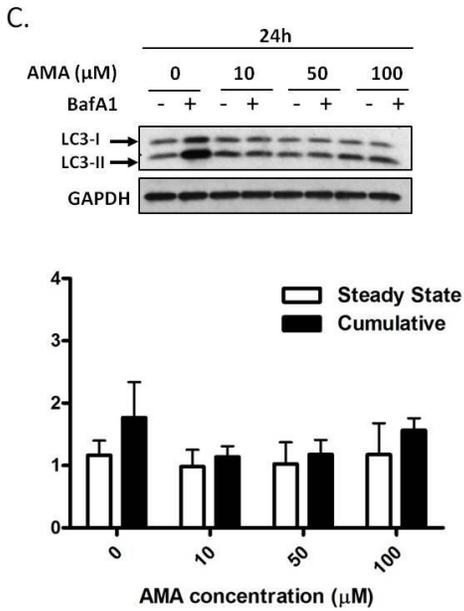
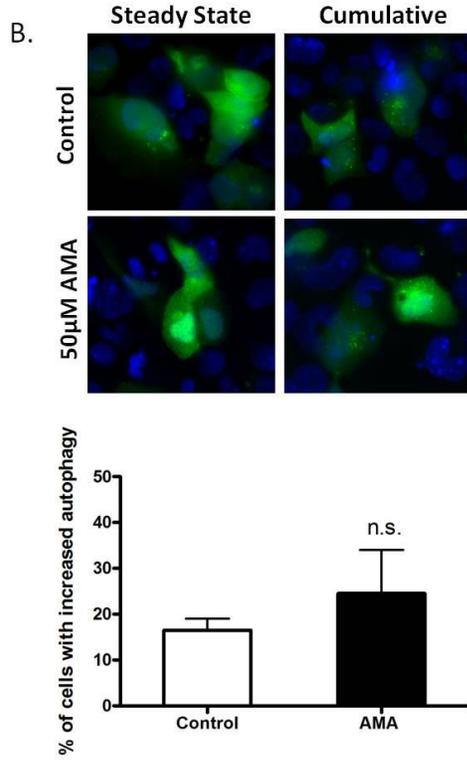
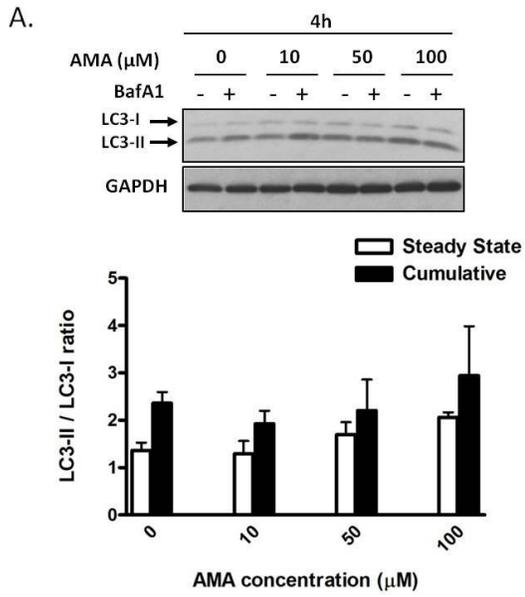


Figure 2-2. Treatment with AMA does not induce autophagy in HL-1 cardiomyocytes. (A) Cells were incubated with increasing concentrations of AMA or vehicle control for 4h, in the absence or presence of 75 nM BafA1. Cells were subsequently lysed, immunoblotted for LC3B and LC3-II/LC3-I ratio calculated to determine steady state or cumulative autophagy. GAPDH is used as a loading control. (B) Cells were transfected with GFP-LC3 plasmid and 24h after transfection, treated with 50 μ M AMA or vehicle control for 4h, in the absence or presence of BafA1. Cells were subsequently fixed in 4% paraformaldehyde, stained with DAPI and images taken using epifluorescence microscope. Representative images of steady state and cumulative autophagy are shown (C) Cells were incubated with increasing concentrations of AMA or vehicle control for 24h, in the absence or presence of 75 nM BafA1 added during the final 2h of incubation. Cells were subsequently lysed, immunoblotted for LC3B and LC3-II/LC3-I ratio calculated to determine steady state or cumulative autophagy. GAPDH is used as a loading control (D) Cells were treated with the indicated concentrations of AMA for 2h or 24h and were subsequently lysed and immunoblotted for Atg5-Atg12, Beclin-1 and Atg7. GAPDH is used as a loading control. Data represents mean \pm SE and is derived from three independent experiments.

(see figure on next page)



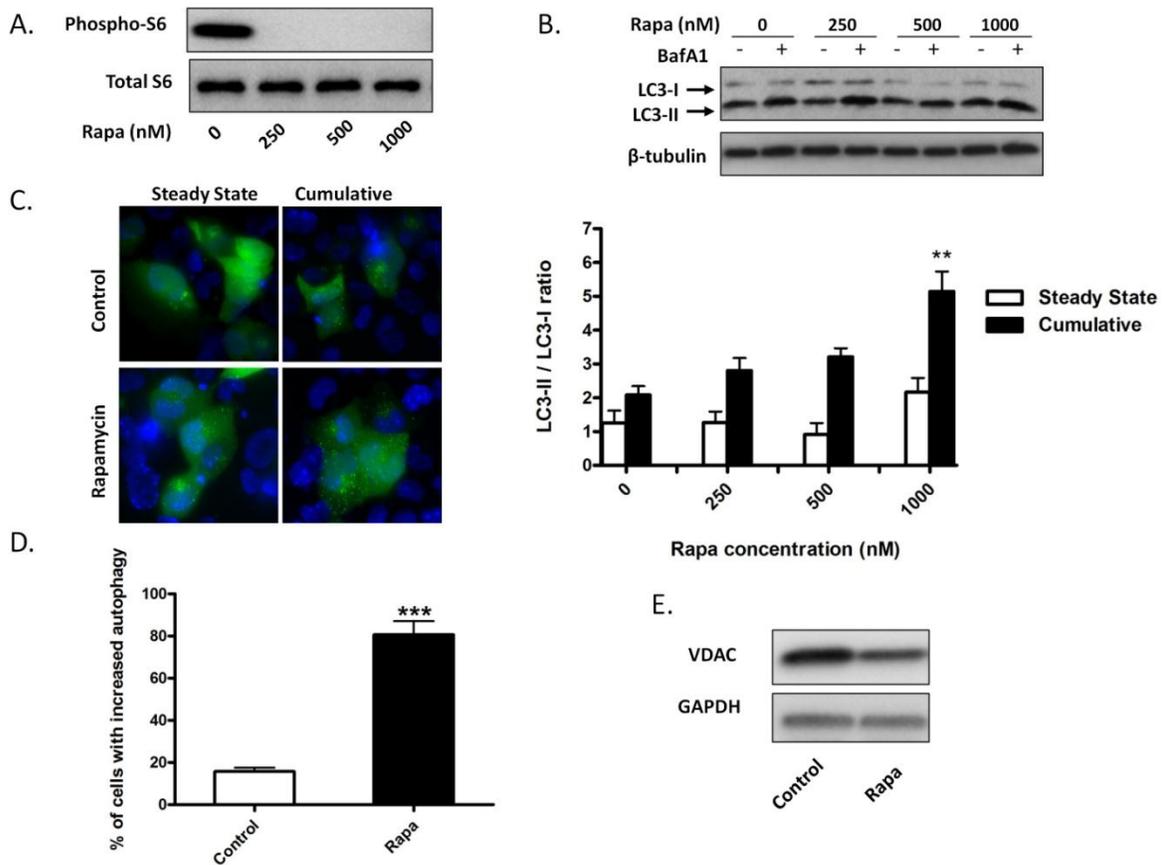
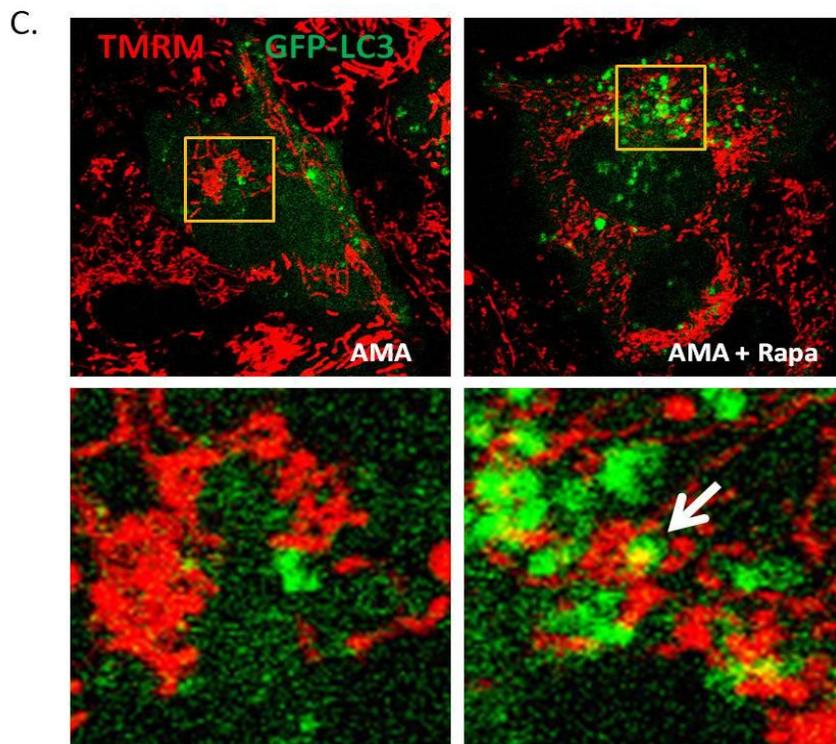
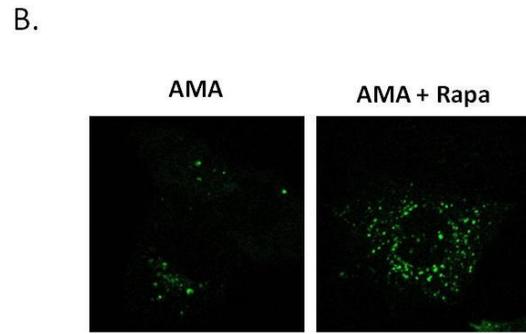
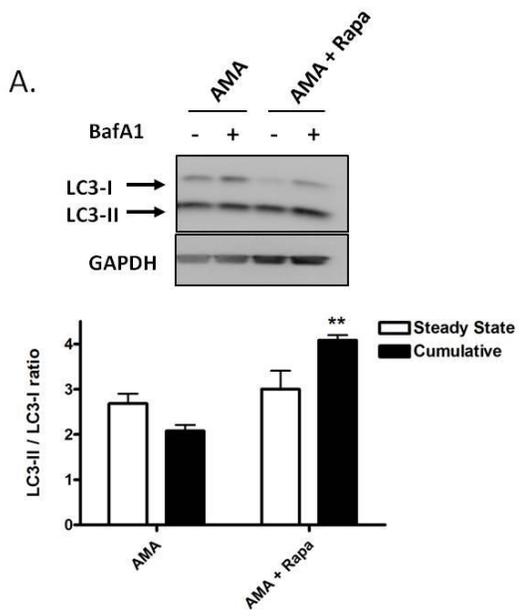


Figure 2-3. Treatment with rapamycin induces autophagy in HL-1 cardiomyocytes. (A) Cells were incubated with increasing concentrations of rapamycin or vehicle control for 16h and subsequently lysed and immunoblotted for phospho-S6 and total S6 levels. (B) Cells were incubated with increasing concentrations of rapamycin or vehicle control for 16h, in the absence or presence of 75 nM BafA1 added during the final 4h of incubation. Cells were subsequently lysed, immunoblotted for LC3B and LC3-II/LC3-I levels calculated to determine steady state or cumulative autophagy. β -tubulin is used as a loading control. ** $p < 0.01$ vs. control. (C) Cells were transfected with GFP-LC3 plasmid and 24h after transfection, treated with 1 μ M rapamycin or vehicle control for 16h, in the absence or presence of BafA1 added during the final 4h of incubation. Cells were subsequently fixed in 4% paraformaldehyde, stained with DAPI and images taken using epifluorescence microscope. Representative images of steady state and cumulative autophagy are shown. (D) The number of GFP-LC3 puncta for cumulative autophagy in (C) were counted in each cell to determine the percentage of cells with stimulated autophagy *** $p < 0.001$ vs. control. (E). Cells were treated with vehicle control or 1 μ M rapamycin for 48h. Cell lysates were subsequently collected and immunoblotted for VDAC. GAPDH is used as a loading control. Rapa, rapamycin. Data represents mean \pm SE and is derived from three independent experiments.

Figure 2-4. Rapamycin induces autophagy in the presence of AMA in HL-1 cardiomyocytes. (A) Cells were treated with 50 μ M AMA or 1 μ M Rapamycin plus 50 μ M AMA for 16h in the absence or presence of 75 nM BafA1 added during the final 4h of incubation. Cells were subsequently lysed, immunoblotted for LC3B and LC3-II/LC3-I ratio calculated to determine steady state or cumulative autophagy. GAPDH is used as a loading control. ** $p < 0.01$ vs. AMA + Baf. (B) Cells were transfected with GFP-LC3 plasmid and 24h after transfection, treated with 1 μ M rapamycin or vehicle control for 16h. Cells were subsequently treated with 50 μ M AMA for a period of 2h and imaged using confocal microscopy. (C) Cells were transfected with GFP-LC3 plasmid and 24h after transfection, treated with 1 μ M rapamycin or vehicle control for 16h. Cells were subsequently incubated with 50 nM TMRM for 30 mins. Cells were finally treated with 50 μ M AMA and imaged using confocal microscopy. Arrow indicates mitochondria surrounded by a growing autophagic vacuole. Rapa, Rapamycin. Data represents mean \pm SE and is derived from three independent experiments.

(see figure on next page)



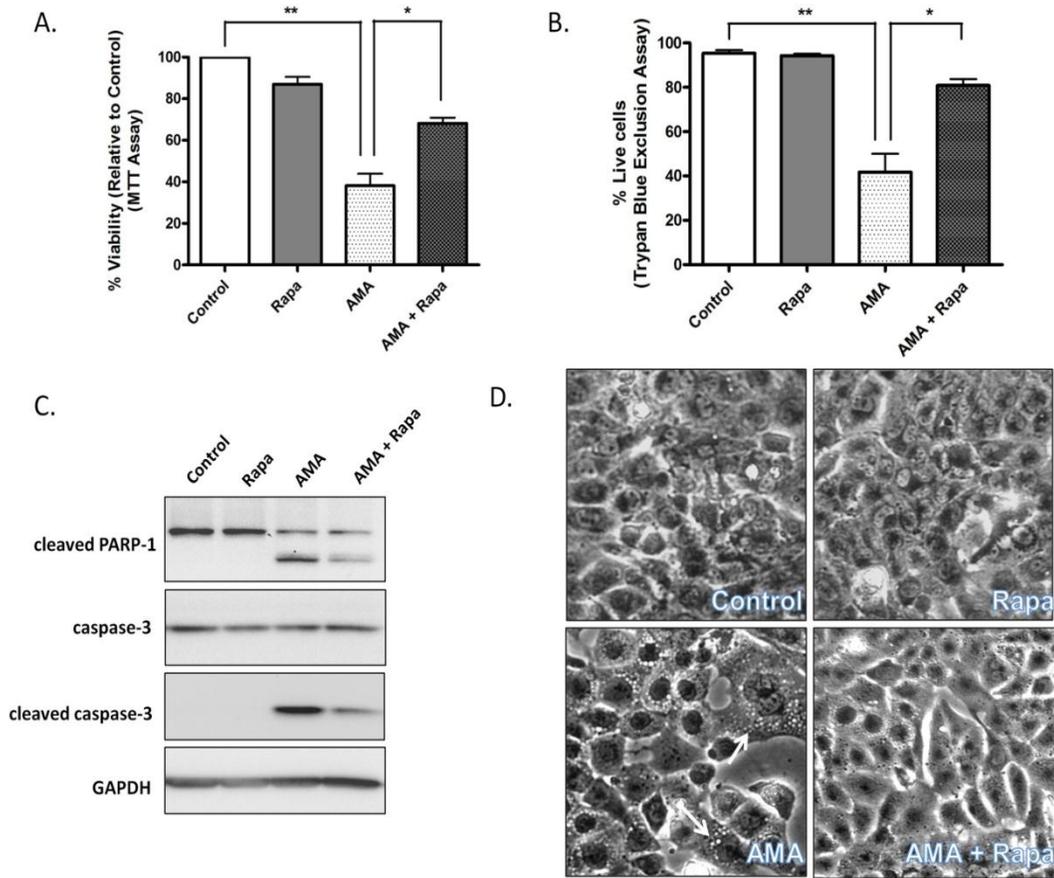


Figure 2-5. Rapamycin protects against the cytotoxic effects of AMA. (A-C) Cells were pre-treated with 1 μ M Rapamycin for 16h, followed by incubation with 50 μ M AMA or vehicle control for an additional 32h, in the absence or presence of rapamycin. Cell viability was subsequently determined using MTT assay (A) and Trypan Blue exclusion assay (B) or cell lysates were collected and immunoblotted for cleaved PARP-1 and caspase-3 protein expression (C). For (A) and (B), data represents mean \pm SE and is derived from three independent experiments. GAPDH is used as a loading control for (C). * p < 0.05; ** p < 0.01 vs. control. (D) Cells were pre-treated with 1 μ M rapamycin for 16h, followed by incubation with 50 μ M AMA or vehicle control for an additional 24h, in the presence of rapamycin. Representative phase contrast images of cells are shown. Rapa, rapamycin.

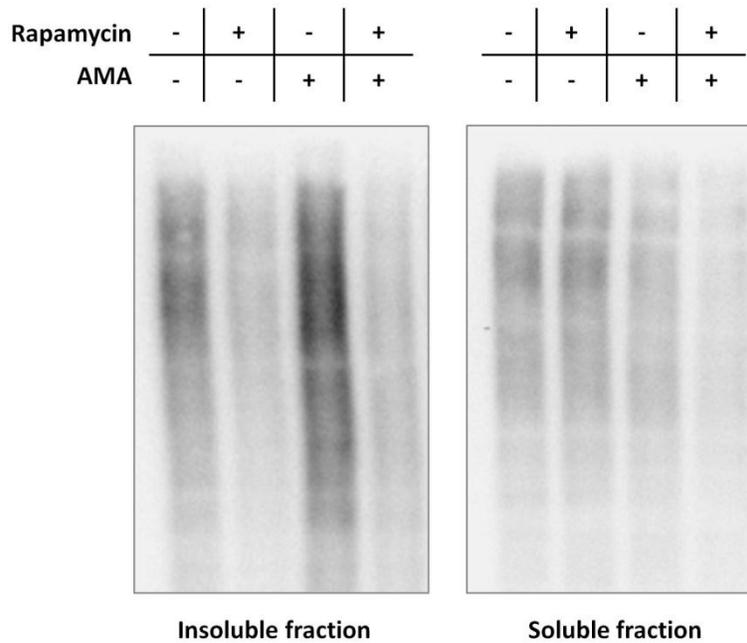


Figure 2-6. Rapamycin enhances the loss of ubiquitinated proteins induced by AMA in HL-1 cardiomyocytes. Cells were pre-treated with 1 μ M rapamycin for 16h, followed by incubation with 50 μ M AMA or vehicle control for an additional 32h, in the absence or presence of rapamycin. Cell lysates were collected and detergent soluble and insoluble fractions were immunoblotted for ubiquitinated proteins. Rapa, rapamycin.

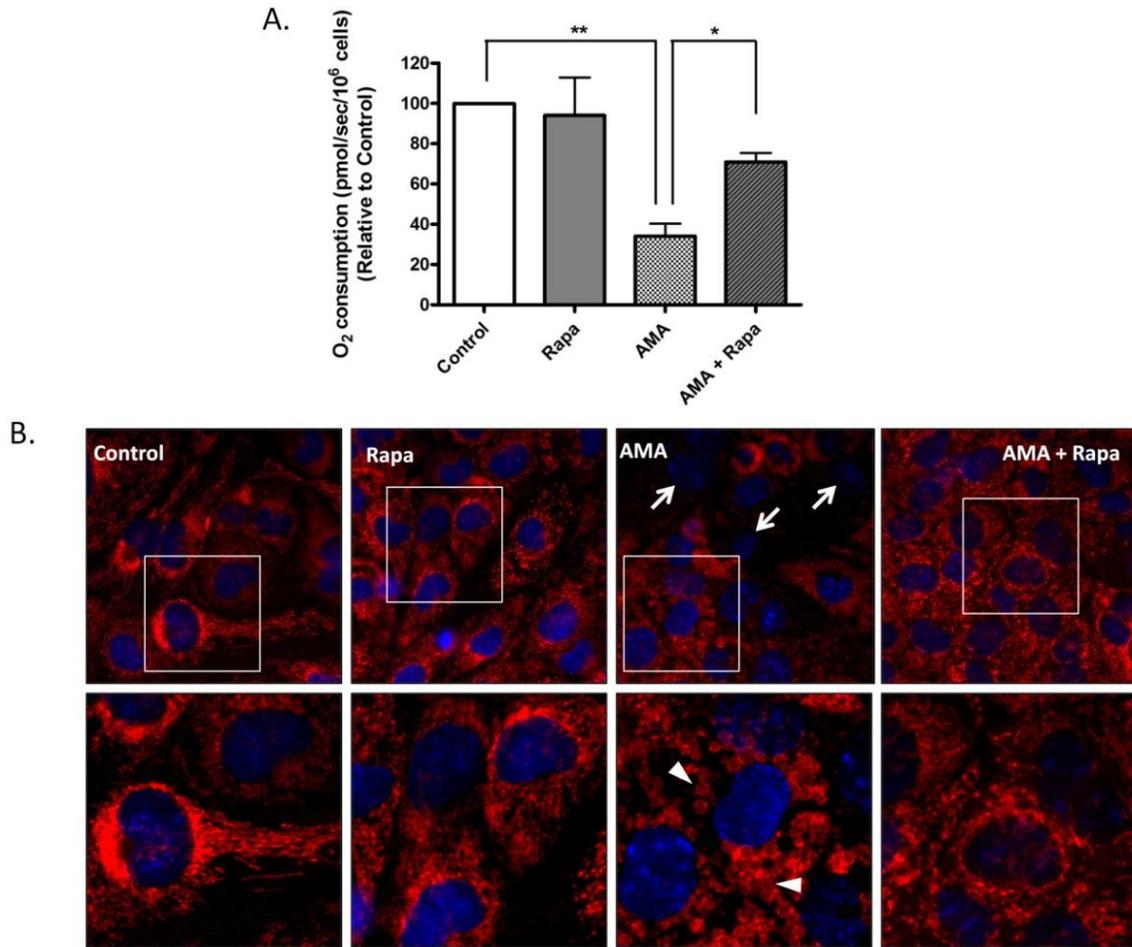


Figure 2-7. Rapamycin protects against AMA-induced mitochondrial depolarization and respiration dysfunction in HL-1 cardiomyocytes. (A) Cells were pre-treated with 1 μ M rapamycin for 16h, followed by treatment with 50 μ M AMA or vehicle control for an additional 24h, in the absence or presence of rapamycin. Cells were subsequently trypsinized, resuspended in Claycomb media and routine respiration analyzed. * $p < 0.05$; ** $p < 0.01$ vs. control. Data represents mean \pm SE and is derived from three independent experiments. (B) Cells were pre-treated with 1 μ M rapamycin for 16h, followed by incubation with 50 μ M AMA or vehicle control for an additional 24h, in the presence of rapamycin. Cells were subsequently stained with 50 nM TMRM (red) and 1 μ g/ml Hoechst 33342 (blue) and imaged using epifluorescence microscopy. Arrows represent cells with no $\Delta\psi_m$. Arrowheads represent swollen mitochondria. Rapa, rapamycin.

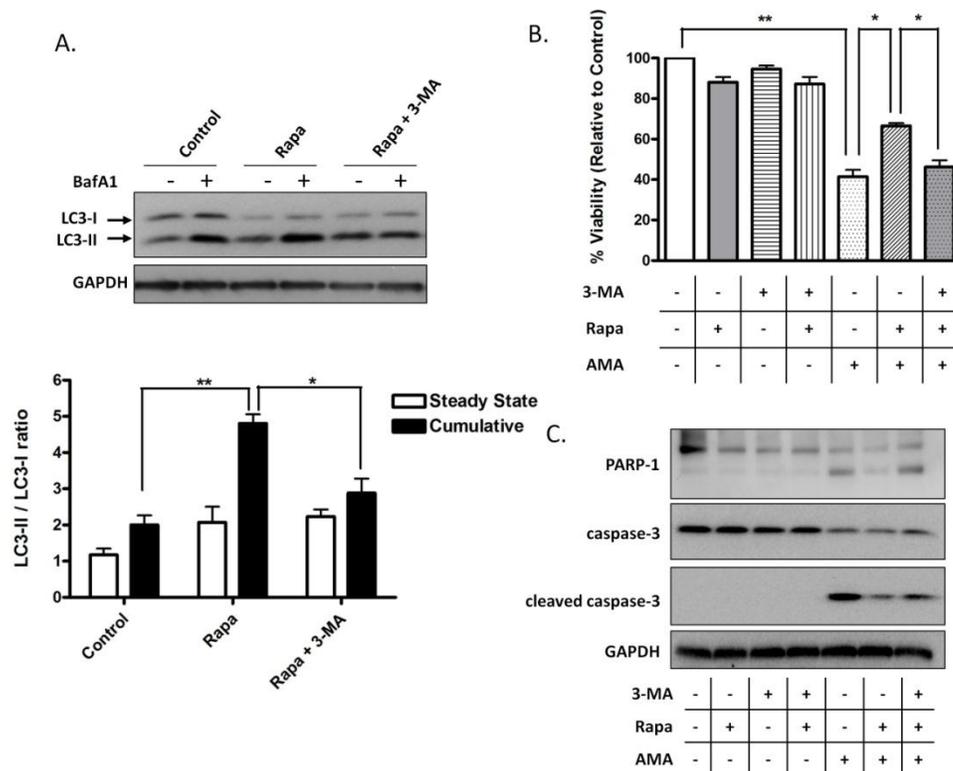


Figure 2-8. Inhibition of autophagy attenuates rapamycin-mediated protection against AMA toxicity in cardiomyocytes. (A) Cells were treated with 1 μ M rapamycin in the absence or presence 5 mM 3-MA for a period of 16h and with or without 75 nM BafA1 added during the final 4h of incubation. Cells were subsequently lysed, immunoblotted for LC3B and LC3-II/LC3-I levels calculated to determine steady state or cumulative autophagy. (B) Cells were pre-treated with 1 μ M rapamycin in the absence or presence of 5 mM 3-MA for 16h, followed by treatment with 50 μ M AMA or vehicle control for an additional 32h, in the presence of rapamycin and 5mM 3-MA. Cell viability was subsequently assessed using MTT assay. (C) Cells were treated as in (B), cell lysates collected and immunoblotted for PARP-1 and caspase-3 protein expression. GAPDH is used as a loading control. Data represents mean \pm SE and is derived from three independent experiments.

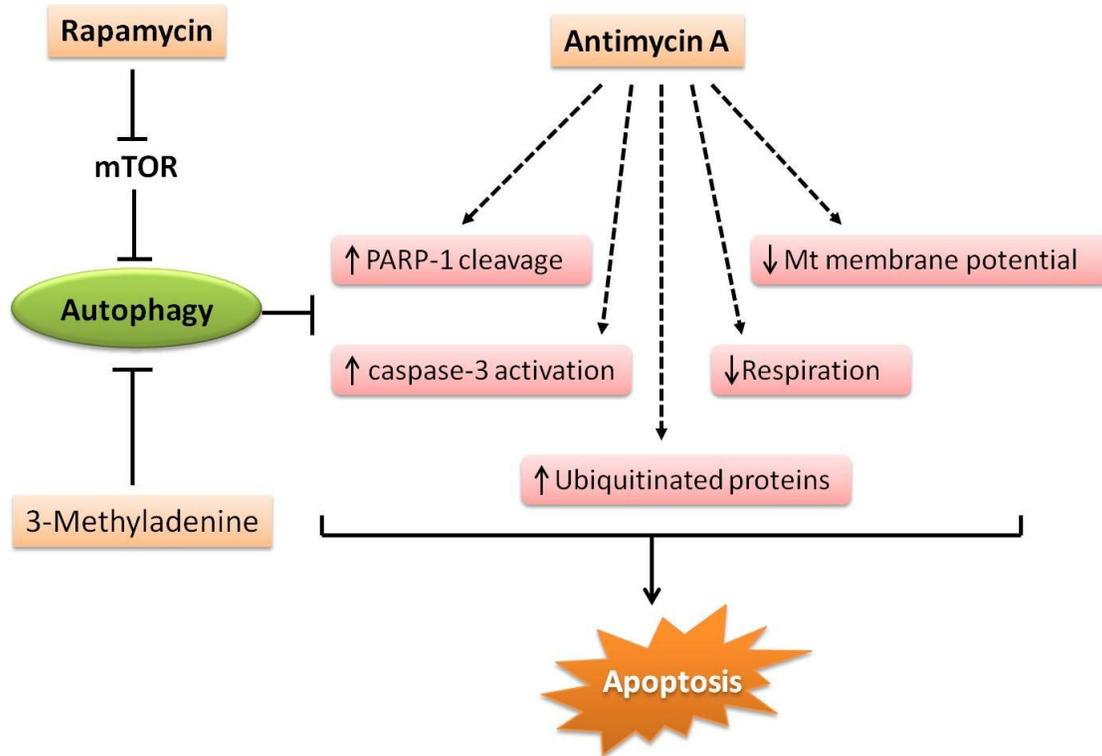


Figure 2-9. Schematic representation of the protective effects of rapamycin against AMA toxicity. AMA induces PARP-1 cleavage and caspase-3 activation, decreases respiration, mitochondrial membrane potential as well as mediates the accumulation of ubiquitinated proteins, all of which culminates in apoptotic induction. Rapamycin treatment induces autophagy by removing the inhibitory effect of mTOR on autophagy induction. Stimulation of autophagy suppresses cytotoxic markers induced by AMA. Inhibition of rapamycin-induced autophagy by 3-MA blocks the cytoprotective effects.

Table 2-1. List of reagents used for in vitro study

Reagent Name	Source	Catalog No.
3-MA	Sigma, St. Louis, MO	M9281
Alkaline phosphatase	Sigma, St. Louis, MO	P6774-10KU
Antimycin A	Sigma, St. Louis, MO	A8674
Bafilomycin A1(BafA1)	Sigma, St. Louis, MO	B1793
Claycomb Media	JRH Biosciences, Lenexa, KS	51800C
DAPI	Vector Laboratories, Burlingame, CA	H-1200
Dc protein assay solution	Biorad, Hercules, CA	500-0113
DFOM (deferoxamine mesylate)	Sigma, St. Louis, MO	D9533
dGuo standards	Sigma, St. Louis, MO	D7154
DMSO (Dimethyl sulfoxide)	Sigma, St. Louis, MO	D2650
ECL Plus reagent	Amersham Biosciences, Buckinghamshire, UK	RPN2132
FBS (Fetal bovine serum)	Sigma, St. Louis, MO	F2442
Fluorodish (0.17mm glass)	World Precision Instruments, Sarasota, FL	FD35-100
Hoechst 33342	Invitrogen, Carlsbad, CA	H3570
L-glutamine	Life Technologies, Gaithersburg, MD	25030-081
Lipofectamine 2000	Invitrogen, Carlsbad, CA	11668-027
MitoSOX Red	Invitrogen, Carlsbad, CA	M36008
MTT	Sigma, St. Louis, MO	M5655
Norepinephrine	Sigma, St. Louis, MO	A0937
Nuclease P1	MP Biomedicals, Solon, OH	N8630
Paraformaldehyde	Electron Microscopy Sciences, Fort Washington, PA	50-00-0
Penicillin / Streptomycin	Life Technologies, Gaithersburg, MD	15140-122
PVDF membrane	Biorad, Hercules, CA	162-0177
Rapamycin	Calbiochem, San Diego, CA	553211
Starting Block	Thermo Scientific, Fair Lawn, NJ	37543
TMRM	Invitrogen, Carlsbad, CA	T668

Table 2-2. List of primary antibodies used for in vitro study

Species	Antigen	Source	Catalog No.
mouse	anti-Atg5	Sigma, St. Louis, MO	A2859
rabbit	anti-Beclin-1	Cell Signaling Technology, MA	3738
rabbit	anti-caspase-3	Cell Signaling Technology, MA	9662
rabbit	anti-LC3B	Cell Signaling Technology, MA	2775
rabbit	anti-PARP-1	Cell Signaling Technology, MA	9542
rabbit	anti-phospho-S6	Cell Signaling Technology, MA	2211
mouse	anti-S6	Cell Signaling Technology, MA	2317
mouse	GAPDH-HRP	Sigma, St. Louis, MO	G9295
rabbit	rabbit anti-ubiquitin	Cell Signaling Technology, MA	3936

CHAPTER 3
CALORIE RESTRICTION COMBINED WITH RESVERATROL INDUCES
AUTOPHAGY AND OFFERS PROTECTION FROM DOXORUBICIN-INDUCED
TOXICITY IN 26-MONTH-OLD RAT HEARTS

Introduction

The housekeeping process of autophagy has been shown to be required for optimal cardiac functioning and survival (Nakai, 2007 and Taneiki, 2007). Given the post mitotic nature of the heart, autophagy-mediated recycling of damaged cellular components is essential for protein and organelle quality control. This is supported by the observation that abrogation of the autophagic pathway in the adult heart by conditional inactivation of Atg5 or Atg7 causes rapid onset cardiac abnormalities characterized by cardiac hypertrophy, left ventricular dilatation and decreased cardiac output (Nakai, 2007 and Taneiki, 2010). In addition, an age-related decline in the efficiency of the autophagic process can lead to the accumulation of damaged cellular components which can further lead to functional deterioration (Terman & Brunk, 2005). Hence, interventions that induce autophagy in vivo, especially at an older age, are highly desirable and potentially beneficial.

Although inducers of autophagy in cell culture conditions are increasingly being reported (reviewed in Chapter 4), few studies have investigated interventions to induce autophagy in vivo, especially, starting at an older age. As regards the heart, Kanamori et al... reported that starving GFP-LC3 transgenic mice for 12h led to an increase in fluorescent autophagic puncta, which was also confirmed by electron microscopic visualization of autophagic vacuoles (Kanamori et al., 2009). Longer starvation periods led to a more robust autophagic stimulation and enhanced the expression of downstream lysosomal enzymes such as cathepsin D, suggesting an overall

enhancement of autophagic flux (Kanamori et al., 2009). Consistent with the abovementioned study, our group has previously shown that lifelong 40% CR can increase the appearance of autophagic vacuoles in the hearts of Fischer 344 rats and enhance the expression of autophagy proteins Atg7 and Atg9 and lysosomal enzyme procathepsin D (Wohlgemuth et al., 2007). Similar effects have also been observed in the skeletal muscle of 40% CR animals (Wohlgemuth et al., 2010). However, in spite of the autophagy enhancing capability of lifelong 40% CR, such a severe dietary restriction is not feasible in humans. Additionally, it can lead to undesirable and potentially harmful changes if started at a vulnerable stage of life cycle, such as in young adolescents, the very old or in pregnant women. Hence in this present study, we investigated whether a moderate CR (20%) regimen, beginning in late-middle-age, can induce autophagy in the rat heart.

In addition to a mild CR regimen, we investigated the natural polyphenol resveratrol as a potential inducer of autophagy. Resveratrol is produced in a wide variety of plants such as grapes, berries and peanuts in response to environmental stress and has been extensively investigated in a large number of clinical studies (Mullin, 2011). Resveratrol has been implicated in the French paradox, an observation of how the French people, in spite of consuming high amounts of saturated fats in their diet, have decreased incidences of age-associated cardiovascular disorders. It is believed that this protective effect is due to their high consumption of red wine, which contains resveratrol. In experimental animals, resveratrol has shown numerous beneficial effects, such as promoting vasodilation in models of coronary heart disease and enhancing the expression of antioxidant enzymes (Bradamante et al., 2004).

Additionally, resveratrol can protect the heart against ischemia-reperfusion injury (Ray et al., 1999), improve endothelial function (Li & Forstermann, 2009) and prevent platelet aggregation (Ramprasath & Jones, 2010). Notably, resveratrol has been shown to be a potent inducer of autophagy in several cell culture models, including cardiomyocytes (Jeong et al., 2012; Lv & Zhou, 2012; Scarlatti et al., 2008; Wu et al., 2011). However, studies investigating whether resveratrol can induce autophagy in animals are limited. Our preliminary analysis on resveratrol-fed rats (5 and 50 mg/kg/day resveratrol for 6 weeks) did not show any changes in cardiac autophagic markers in 26-month-old Fischer 344 x Brown Norway (FBN) rats (data not shown). Therefore, in the present study, we investigated whether resveratrol combined with 20% CR is able to induce autophagy in the hearts of 26-month-old FBN rats.

In addition to exploring interventions to induce autophagy in rodent hearts, we further investigated whether such autophagy interventions can protect against cardiac damages induced by the oxidative stressor, doxorubicin. Doxorubicin is a highly effective chemotherapeutic agent used in the treatment of solid tumors and hematologic malignancies (Octavia et al., 2012). However, the cardiotoxic effects of doxorubicin severely limit its chemotherapeutic efficacy (Childs et al., 2002; Octavia et al., 2012). Although a complete understanding of the mechanisms involved in doxorubicin's toxicity remain elusive, the generation of ROS in cells has been proposed to be a major player (Mordente et al., 2012; Takemura & Fujiwara, 2007). Doxorubicin has been reported to localize to the mitochondria, induce ultrastructural changes and affect mitochondrial function (Green & Leeuwenburgh, 2002; Montaigne et al., 2010). Doxorubicin contains an unsubstituted quinone ring, which can act as an electron acceptor in reactions

mediated by oxidoreductase enzymes in the cell, such as by the NADH dehydrogenase (Doroshov, 1983; Mukhopadhyay et al., 2009). The addition of free electrons converts quinone to semiquinone, which can subsequently interact with molecular oxygen and generate $O_2^{\cdot-}$. The generation of ROS by doxorubicin can lead to cellular oxidative damage and activation of proteases, resulting in cytotoxic effects (Kotamraju et al., 2000).

Our study shows that 50 mg/kg/day resveratrol combined with 20% CR can enhance autophagic flux in the hearts of 26-month-old FBN rats. In addition, such an induction of autophagy has a beneficial effect on doxorubicin-mediated toxicity. We therefore propose that induction of autophagy in late middle-aged rat hearts could be developed as a therapeutic target for mitigating oxidative stress-induced damage.

Materials and Methods

Animals and Experimental Design

Male FBN rats at 25 months of age were purchased from the National Institute on Aging (NIA) and were singly housed at the University of Florida animal facility in a temperature ($20 \pm 2.5^{\circ}C$) and light-controlled (12:12 h light-dark cycle) environment with unrestricted access to water. After arrival, animals were acclimated for a period of 3 weeks, during which period their baseline food consumption was measured. At the end of the acclimation period, rats were randomly assigned into one of four experimental groups: 1) Ad libitum (AL) 2) 20% CR (CR), 3) CR with 5 mg/kg/day resveratrol (CR + Resv-5) and 4) CR with 50 mg/kg/day resveratrol (CR + Resv-50). They were maintained on the interventions for a period of 6 weeks. Animals on the CR, CR + Resv-5 and CR + Resv-50 groups received 20% less food from a 20% fortified diet so that they were not compromised on the amino acid, vitamins and minerals intake. The diet

was based on AIN-93M chow, formulated for the maintenance of mature rodents and have reduced levels of proteins and fats to help reduce the incidence of kidney stones in older rodents (Reeves et al., 1993). The composition of the AL and the CR diet has been summarized in Table 3-1. Resveratrol (Sigma, St. Louis, MO) was given as highly palatable supplementation pellets, prepared by Custom Animal Diets (Bangor, PA) utilizing an effective bacon flavor masking capability. To ensure that every rat received the proper dose of resveratrol, the number of pellets given on each day was calculated based on the body weight of the animal. Control supplementation tablets (without resveratrol) was also given to AL rats. The supplementation tablets were similarly formulated with AL or CR diet as the base chow, with or without resveratrol. Throughout the entire study, the amount of food given to the CR rats was adjusted weekly based on the food consumed by AL rats the week prior. Body weights of rats were recorded at least once per week.

At the end of the intervention period, animals received a single i.p. injection of 10 mg/kg doxorubicin (Sigma, St. Louis, MO) or saline control. The dose was chosen based on our previous observation that 10 mg/kg/day doxorubicin can activate caspase-3 and induce apoptosis in the hearts of Fisher 344 rats (Jang et al., 2004). Twenty-four hours after injection, animals were sacrificed by rapid-decapitation. Serum and plasma was collected by trunk blood processing and stored at -80°C until further analysis. All experiments and procedures were approved by the Institute on Animal Care and Use Committee at the University of Florida.

Preparation of Tissue for Histological Staining

Upon sacrifice, the heart was removed, rinsed in DPBS, blotted dry, weighed and dissected into individual compartments. Approximately 10 mg of the left ventricle was

further dissected out and placed immediately in O.C.T. compound (Fisher Scientific, CA) in 15 x 15 x 5 mm cryomolds (Fisher Scientific, CA). The tissue was subsequently snap frozen in liquid nitrogen and stored at -80°C until further analysis. Hematoxylin and eosin (H&E) staining on O.C.T embedded tissue was performed at the Cell and Tissue Analysis Core (CTAC) at the University of Florida and images were taken using Zeiss Axio Observer A1 microscope (Zeiss, Germany) at 20X objective magnification.

Body Composition Analysis

Fat and lean mass percentages of rats were determined at baseline levels (before the start of interventions) and after 6 weeks of CR and resveratrol interventions using time domain-nuclear magnetic resonance (TD-NMR) analyzer (Minispec, Bruker Optics, TX). TD-NMR provides an accurate, fast, and easy-to-use method for determining fat and lean tissue in rodents without the need for anesthesia. The validation of the TD-NMR methodology has been provided elsewhere (Nixon et al., 2010).

Preparation of Tissue Extracts

For the preparation of tissue extracts, approximately 100 mg of the left ventricle was pulverized in liquid nitrogen. The powder was subsequently resuspended in 1 mL ice-cold buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β -glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4 , 10% glycerol, 1% Triton X-100 and Halts Protease Inhibitor (Thermo Scientific, NJ). Resuspended tissue was further homogenized using a mechanically driven Potter-Elvehjem glass-glass homogenizer with approximately 14 up and down strokes. The homogenates were subsequently rotated for 1h at 4°C for efficient cell lysis. Finally, the homogenates were centrifuged at 10,000g for 10 minutes at 4°C and the supernatant was transferred to clean tubes.

Protein concentrations were determined using Bradford Assay (Bradford, 1976) and aliquots were stored at -80⁰C until further analysis. A complete list of all the reagents used in the study and their catalog numbers have been provided in Table 3-2.

Western Blotting

Protein samples were prepared in Laemmli buffer (62.5 mM Tris-HCl, 2% SDS, 25% Glycerol, 0.01% Bromophenol Blue, pH 6.8; BioRad, CA) with 5% β -mercaptoethanol and were boiled at 95⁰C for 5 mins prior to loading in gels. Equal amounts of proteins were loaded in pre-cast Tris-HCl polyacrylamide gels (Criterion system, BioRad, Hercules, CA). After electrophoretic separation, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA) and subsequently blocked for 1h in Starting Block (Thermo Scientific, Fair Lawn, NJ), followed by overnight incubation with primary antibodies at 4⁰C. Membranes were subsequently washed with Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with the appropriate secondary antibodies in Starting Block for 1h. Membranes were washed again with TBST, chemiluminescent signals developed using ECL Plus reagent (Amersham Biosciences, Buckinghamshire, UK) and captured using ChemiDoc XRS System (BioRad, CA). Digital images were analyzed for densitometry using ImageLab software (BioRad, CA). The primary antibodies used for immunoblotting analysis are as follows: rabbit anti-LC3B (1:1000), rabbit anti-p62 (1:1000) rabbit anti-Beclin-1 (1:1000) (all from Cell Signaling Technology, MA), mouse anti-Atg5-Atg12 conjugate (1:1000; Sigma, MO) and GAPDH-HRP (1:35,000; Sigma, MO). HRP conjugated secondary antibodies were anti-rabbit (1:10,000; GE-Amersham,

IL) or anti-mouse (1:10,000; GE-Amersham, IL). A complete list of primary antibodies and their catalog number have been provided in Table 3-3.

Plasma Resveratrol Concentration Analysis

Trans-resveratrol was quantified in rat plasma using LC/MS/MS assay at the Biomedical Mass Spectrometry Core at the University of Florida. Rat serum samples (30 μ L) were loaded onto an Accela Open Autosampler (Thermo Fisher Scientific, CA) connected to an Accela 600 UPLC and LTQ Velos mass spectrometer (Thermo Fisher Scientific, CA). Separation was achieved on a BetaBasic C18 HPLC column (150 x 2.1 mm, 5 μ m, ThermoFisher, CA).

Blood Glucose Analysis

Glucose levels were measured in freshly collected trunk blood using blood glucose meter and test strips (both from Ascensia Contour; Bayer HealthCare LLC).

Apoptotic Analysis

Apoptotic analysis on left ventricular homogenates was performed using Cell Death Detection Plus ELISA kit (Roche Diagnostics, IN) following manufacturer's instructions. The kit is designed to detect mono- and oligonucleosomes in the cytoplasmic fraction of heart lysates and is based on a sandwich-enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. Absorbance was read at 405 nm with a Biotek Synergy plate reader (Biotek, VT) and the values were normalized to protein concentrations.

Creatine Kinase (CK) Assay

CK levels in serum were determined using Enzychrom Creatine Kinase Assay kit (Bioassay Systems, CA) following manufacturer's instructions. The kit utilizes an enzyme-coupled reaction in which creatine phosphate and ADP is converted to creatine

and ATP by CK. The ATP generated in the process is used by hexokinase in phosphorylating glucose to glucose-6-phosphate. The latter is subsequently oxidized by NADP (in the presence of glucose-6-phosphate dehydrogenase) and produces NADPH in the process. The produced NADPH is proportional to the CK activity in the samples and is quantified by measuring the absorbance at 340 nm using Biotek Synergy plate reader (Biotek, VT).

Lactate Dehydrogenase (LDH) Assay

LDH levels in serum were measured using Quantichrom Lactate Dehydrogenase Assay kit (BioAssay Systems, CA) according to manufacturer's instructions. The assay is based on NADH catalyzed reduction of the tetrazolium salt MTT to a reduced form. Absorbance is measured at 565 nm using Biotek plate reader (Biotek, VT) and the values are directly proportional to the enzyme activity.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism Version 4.0 (GraphPad Software, San Diego, CA). Student's t-test and one-way ANOVA were performed, wherever applicable. Whenever required, post-hoc analysis was performed using Bonferroni multiple comparison test. Statistical significance was set to $p < 0.05$. Results are expressed as mean \pm SE.

Results

CR Diet were Associated with Lower Body Weights

Six weeks of 20% CR, with or without resveratrol, resulted in significantly lower body weights in comparison to AL ($p < 0.001$ vs. AL, Table 3-4). Heart weights of rats in CR + Resv-5 and CR + Resv-50 groups were significantly lower in comparison to that of

AL rats ($p < 0.001$ vs. AL). There was no statistical difference when heart weight was normalized to body weight (Table 3-4).

Six weeks of CR and Resveratrol Interventions or Administration of Doxorubicin does not Change Myocardial Morphology

Since we observed decreased heart weights in animals fed CR + 5 mg/kg/day or CR + 50 mg/kg/day resveratrol, we performed H&E staining on frozen left ventricular tissue sections to determine whether the interventions affected cardiac morphology, such as cardiac fibrosis or increased cardiomyocyte dropout. We observed no differences between AL groups and the CR + Resv-5 or CR + Resv-50 groups by histology analysis (Fig. 3-1, upper panel). Doxorubicin administration for 24h also did not change tissue morphology (Fig. 3-1, lower panel).

CR Diet alone or in Combination with Resveratrol, Attenuates Body Composition Changes over Time

Paired t-test analysis showed that fat mass % and fat / lean ratio significantly increased ($p < 0.01$) and lean mass % decreased ($p < 0.05$) in animals on AL diet over a 6-week period (Fig. 3-2). However, fat mass %, lean mass % and fat / lean ratio remained unchanged in the CR, CR + Resv-5 and CR + Resv-50 groups before and after the intervention period.

Plasma Resveratrol Concentrations Increase in a Dose-Dependent Manner in Resveratrol-Fed Rats

Plasma resveratrol concentrations determined by LC/MS/MS analysis showed a dose-dependent increase in resveratrol concentrations (Table 3-5). The mean plasma concentration of resveratrol in the oral administration doses of 5 and 50 mg/kg/day was 7.4 ± 3.7 and 41 ± 19 ng/mL, respectively. Resveratrol could not be detected in the plasma of AL rats (Table 3-5).

Doxorubicin Administration Increases Blood Glucose Levels

Doxorubicin administration has been reported to increase blood glucose levels, primarily due to damage to insulin-secreting beta cells in the pancreas (Deleers & Goormaghtigh, 1985). We observed that blood glucose levels were increased in animals treated with doxorubicin (main effect, $p < 0.001$ vs. saline, Table 3-6). None of the interventions were able to attenuate the increase in blood glucose levels, although animals in the CR + Resv-50 group had the lowest blood glucose levels (118 ± 30 mg/dL), close to that of AL rats (112 ± 17 ng/dL).

CR + 50 mg/kg/day Resveratrol Increases Autophagic Flux in the Left Ventricle

We analyzed the expression levels of key autophagy proteins (LC3B, p62, Beclin-1 and Atg5-Atg12 conjugate) in the left ventricle of saline-treated rats, to determine whether 20% CR by itself, or in combination with the two different doses of resveratrol can induce cardiac autophagy in 26-month-old FBN rats. In addition, since oxidative stress per se has been proposed to be an inducer of autophagy, we also determined whether doxorubicin can affect the autophagic process. The results from autophagy protein expression analysis in both saline and doxorubicin-treated groups are summarized below:

LC3B

Autophagy induction converts cytosolic LC3-I to autophagosomal membrane-associated and PE-conjugated LC3-II. LC3-II/LC3-I ratio is therefore a widely used indicator of steady state autophagic levels. We could not detect an increase in LC3-II/LC3-I ratio in any of the intervention groups (Fig. 3-3A). Treatment with doxorubicin however, enhanced LC3-II /LC3-I ratio, in comparison to saline treated AL rats ($p < 0.005$, Fig. 3-4A).

p62

Although LC3-II /LC3-I ratio remained unchanged with the interventions, we cannot rule out the possibility that an increase in the rate of their degradation in the lysosomes, i.e. an enhancement in autophagic flux may have contributed to their unaltered levels. To determine autophagic flux, we measured the abundance of p62 / sequestosome 1 (SQSTM1) in left ventricular homogenates. p62 binds to ubiquitinated proteins through its UBA domain and can interact with LC3 through its LC3 binding motif, thereby directing the degradation of ubiquitinated proteins through the autophagic pathway (Bjorkoy et al., 2009). In the process, p62 is degraded through autophagy; therefore, a decrease in the abundance of p62 is considered indicative of enhanced autophagic flux (Bjorkoy et al., 2009; Larsen et al., 2010). We observed a decrease in p62 abundance in the left ventricle of CR + Resv-50 rats ($p < 0.05$ vs. AL, Fig. 3-3B), indicating an increased autophagic flux. No significant changes were observed in the CR or CR + Resv-5 groups in comparison to AL. Notably, we observed an increase in p62 accumulation in the doxorubicin-treated hearts, suggesting an impairment of the autophagic flux with doxorubicin treatment ($p < 0.01$ vs. saline, Fig. 4-4B).

Beclin-1

Beclin-1 (mammalian homolog of yeast Atg6) is required for the induction phase of autophagy (Liang et al., 1999). Specifically, Beclin-1 associates with a pre-autophagosomal complex comprising of Atg14, Class III PI3K/Vps34 and Vps15 and this complex is required for the localization of autophagic proteins to the pre-autophagosomal structure (Kang et al., 2011; Kihara et al., 2001; Liang et al., 1999). Autophagic triggers have been shown to upregulate Beclin-1 (Wang, 2008). We observed that the expression of Beclin-1 was increased in left ventricular homogenates

of CR + Resv-50 rats in comparison to AL ($p < 0.05$, Fig. 3-3C). There were no changes in Beclin-1 protein levels between saline and doxorubicin treatment groups (Fig. 3-4C).

Atg5-Atg12

In addition to the Atg8-PE (LC3-PE) conjugation system, a second ubiquitin-like conjugation system (comprising of Atg7, Atg12, Atg10) links the carboxy-terminal glycine residue of Atg12 to a lysine of Atg5 (Mizushima et al., 1998). The Atg5–Atg12 conjugate further complexes with Atg16, which is associated with the growing phagophore (Mizushima, 2007). We observed no differences in Atg12-Atg5 levels in left ventricular homogenates between AL rats and the intervention groups (Fig. 3-3D).

CR + 50 mg/kg/day Resveratrol Attenuates Doxorubicin-Mediated Increases in Cardiac Apoptotic Levels

We next determined whether induction of autophagy has a beneficial effect against doxorubicin mediated toxicity in the cardiac tissue. Apoptotic levels were determined by measuring mono- and oligo-nucleosomes released in the cell cytoplasm. Doxorubicin increased apoptotic index in the left ventricle of AL rats ($p < 0.05$ vs. saline, Fig. 3-5A) and CR + Resv-50 attenuated the apoptotic induction ($p < 0.05$ vs. AL + doxorubicin). No significant changes in cardiac apoptotic levels were observed between AL and CR or CR + Resv-5 groups (Fig. 3-5A).

CR + 50 mg/kg/day Resveratrol Attenuates Doxorubicin-Mediated Increases in Serum CK Levels

CK, an enzyme predominantly expressed in muscles, is involved in the conversion of creatine to phosphocreatine with the consumption of ATP (Brancaccio et al., 2010). An elevated CK level in blood is a widely used indicator of muscle damage. We observed that treatment with doxorubicin increased serum CK levels ($p < 0.01$ vs.

saline) and that CR + Resv-50 significantly ameliorated the increase ($p < 0.05$ vs. AL + doxorubicin, Fig. 3-5B).

CR + Resveratrol Attenuates Doxorubicin-Mediated Increases in Serum LDH Activity

LDH is an oxidoreductase enzyme which catalyzes the inter-conversion of lactate and pyruvate. It is usually sequestered within cells and is released into the bloodstream under conditions of tissue injury. Therefore, serum LDH activity represents a marker of general tissue damage (Glick, 1969). We observed that treatment with doxorubicin significantly increased serum LDH levels ($p < 0.01$, Fig. 3-6A) and that both CR + Resv-5 and CR + Resv-50 significantly ameliorated the increase ($p < 0.05$ vs. AL + Dox, Fig. 3-6A). No differences were observed in serum LDH activity among AL and intervention groups in saline-treated rats (Fig. 3-6B).

Discussion

The self-cleanup process of autophagy recycles damaged and dysfunctional cellular components, thereby contributing to organelle and protein quality control. Upregulation of this process could therefore be hypothesized to be beneficial against oxidative stress-induced damage. Despite the beneficial effects of autophagy, few studies have focused on its stimulation in vivo. Although lifelong 40% CR has been reported to enhance autophagy in different tissues (Wohlgemuth et al., 2007; Wohlgemuth et al., 2010), it is likely that most humans will not be able to sustain drastic food restrictions for the long term. Severe CR is associated with osteoporosis, cold sensitivity, slower wound healing, and psychological conditions such as food obsession, depression, and irritability (Marzetti et al., 2009). Furthermore, adolescents practicing chronic severe CR may experience several adverse events such as undesired changes

in physical appearance, loss of strength and stamina, menstrual irregularities, infertility and loss of libido (Marzetti et al., 2009). Hence a moderate CR, starting late in life could offset some of the limitations associated with life-long CR. Additionally, combining a mild CR regimen with a so-called CR mimetic has the potential of reinforcing the beneficial effects of CR, without the potential harmful effects of a drastic food reduction. Resveratrol is a known CR mimetic, which has shown to produce CR-like extension in lifespan in lower organisms and has a proposed mechanism of action very similar to that of CR (Dolinsky & Dyck, 2011; Howitz et al., 2003; Wood et al., 2004).

Although showing numerous CR-like effects, a major drawback of resveratrol is its poor bioavailability. Resveratrol undergoes rapid metabolism to glucoronide and sulphate conjugates in the liver and clearance by the kidney (Kapetanovic et al., 2010), resulting in low bioavailability. For instance, in humans, a single oral dose of 25 mg resveratrol resulted in plasma resveratrol concentrations of < 5 ng/mL (Walle et al., 2004) and similar observations have been reported in rodents (Kapetanovic et al., 2010). However, clinical trials have reported that increased concentrations of resveratrol can be obtained with multiple dosing (Brown et al., 2010; la Porte et al., 2010). In the present study, feeding rats with 50 mg/kg/day resveratrol for a period of 6 weeks resulted in plasma resveratrol concentration of 41 ng/mL (Table 3-5). In this respect, a previous study has reported plasma resveratrol concentrations of 176 ng/mL in rats, given daily oral gavages of 50 mg/kg/day resveratrol for 2 weeks. The study showed that peak plasma resveratrol concentrations were reached 25 mins after the last oral dosing and continued to drop thereafter. The relatively lower plasma resveratrol concentrations in 50 mg/kg/day resveratrol fed rats in the current study can be attributed

to the fact that blood was collected 24h after the last oral dosing. Additionally, we observed dose-dependent increases in plasma resveratrol concentrations, which have also been reported elsewhere (Boocock et al., 2007).

In the present study, we have shown that 20% CR in combination with 50 mg/kg/day resveratrol can enhance autophagic flux in the hearts of late-middle-aged (26-month-old) FBN rats. FBN rats are an excellent model for gerontology research, due to their reduced incidences of age-associated pathological lesions and a relatively longer disease-free life span in comparison to other rat strains (Lipman et al., 1996; Weindruch & Masoro, 1991). In the FBN rats, changes in cardiomyocyte volume density occurs as early as 24 months of age and percentage fractional shortening (as a measure of cardiac function) shows significant differences starting at 27 months of age (Hacker et al., 2006; Walker et al., 2006). These observations suggest that for the FBN rat strain, 26 month may represent an optimum life stage to start late-life interventions, i.e. at a time when structural changes begin to appear in the heart, without drastic functional consequences. Additionally, 26 months of age in FBN rats correspond to roughly 60 years in humans, a stage in life when moderate dietary restriction can be assumed to have little or no deleterious effects (Quinn, 2005).

Autophagy assessments in the study were done by measuring protein expression levels of LC3B, p62, Beclin-1 and Atg5-Atg12 conjugate. We did not observe any changes in the LC3-II /LC3-I ratio in the left ventricle of AL and any of the interventions, which could either mean: 1) no autophagic stimulation by the treatments or 2) an enhanced autophagic flux, such that an increased amount of LC3-II is rapidly degraded in the lysosomes, resulting in unaltered LC3-II /LC3-I ratios. The ubiquitin binding

protein p62 / SQSTM1 targets cytosolic cargo for autophagic degradation, being selectively incorporated into autophagosomes in the process and efficiently degraded in the autolysosomes (Bjorkoy et al., 2005). Therefore, the cellular abundance of p62 inversely correlates with autophagic flux (Mizushima et al., 2010). In our study, CR + Resv-50 rats showed a decrease in the abundance of p62 in the left ventricle, suggesting an increase in autophagic flux in this intervention group. In addition, we observed an increase in Beclin-1 levels in the same rat group, pointing to an enhanced induction of autophagy. Finally, there were no differences in Atg5-Atg12 conjugate levels between AL and any of the intervention groups. Although the conjugation of Atg5 to Atg12 is essential for autophagy, Atg5-Atg12 dissociates from the autophagosome after it completely engulfs the cargo (Mizushima, 2007). Hence, the observation that the Atg5-Atg12 levels remain unaltered is consistent with LC3-II/LC3-I ratios observed in the study.

The exact mechanism by which the combined regimen of 20% CR and 50 mg/kg/day resveratrol (but not either intervention alone) can induce autophagy in rodent hearts remains unknown. Resveratrol is a known CR mimetic and is proposed to have a mechanism of action similar to that of CR (Dolinsky & Dyck, 2011; Korolchuk et al., 2009). It is possible that when present together, they reinforce and activate certain signal transduction pathways, which can lead to an induction of autophagy. For example, the NAD⁺ dependent deacetylase SIRT1 can deacetylate essential autophagy proteins Atg5, Atg7 and Atg8 and activate autophagy (Lee et al., 2008). Both CR and resveratrol has been shown to activate Sir2 (homolog of mammalian SIRT1) in lower organisms (Guarente & Picard, 2005; Howitz et al., 2003) and SIRT1 in mammals

(Chen et al., 2005; Lagouge et al., 2006). However, although activation of the SIRT1 pathway was originally proposed to be responsible for its lifespan extending properties of resveratrol (Howitz et al., 2003; Wood et al., 2004), recent studies have shown inconsistent results (Bass et al., 2007; Kaeberlein et al., 2005). In addition, we have previously shown that oral administration of low-dose resveratrol (4.9 mg/kg/day) starting at middle-age in mice, was unable to activate SIRT1 in any of the analyzed tissues (heart, skeletal muscle, liver and brain) (Barger et al., 2008b), despite the fact that both CR and resveratrol showed very similar gene expression profiles in the hearts in the same study (Barger et al., 2008b; Edwards et al., 2010). Other common mechanism of action include activation of the AMPK pathway (Dasgupta & Milbrandt, 2007), which has been shown to enhance autophagic induction by phosphorylating ULK1 and activating the ULK1-Atg13- Atg17 complex (Kim et al., 2011). Finally, both CR and resveratrol can inhibit the mTOR pathway, which has inhibitory effects on autophagic induction (Hansen et al., 2007; Kapahi et al., 2004; Liu et al.).

Our results also suggest that doxorubicin per se can increase LC3-II/LC3-I ratio. An increase in LC3-II/LC3-I ratio by doxorubicin treatment has previously been reported (Manov et al., 2011; Pan et al., 2011; Smuder et al., 2011), however autophagic flux analysis was not conducted in these studies. We observed that doxorubicin increased p62 abundance in the left ventricle, suggesting that the increase in LC3-II/ LC3-I levels is due to an impairment of autophagic flux. Consistent with this hypothesis, a decrease in the activity of lysosomal enzyme cathepsin D have been reported in the hearts of doxorubicin-treated animals (Gebbia et al., 1985). Additionally, it is possible that the autophagic flux impairment is a characteristic of the relatively older population of rats

used in the study, as all previous studies with doxorubicin have been conducted in a younger cohort (Smuder et al., 2011). For instance, it is possible that doxorubicin induces autophagy as a protective mechanism in all age groups, but complete degradation of autophagic cargo is affected / attenuated only in older rats.

In the clinical setting, a significant proportion of patients administered with doxorubicin for long-term periods go on to develop cardiac pathologies; however, as of yet, there is no specific treatment for preventing such chemotherapy-derived cardiomyopathy (Tokarska-Schlattner et al., 2006). The development of cardioprotective agents that can intervene against doxorubicin-mediated toxicity is therefore highly desirable. Notably, in the present study, we observed that induction of autophagy protects the myocardium from the anthracycline-mediated injury, confirmed by the attenuation of doxorubicin-mediated increases in myocardial apoptotic index and serum CK levels (Fig. 3-5). Although not a specific marker of myocardial damage, CK levels are routinely measured in emergency patients with chest pain. While skeletal muscle injury cannot be ruled out, we believe that the increases in CK levels in doxorubicin-treated rats is primarily due to cardiac damage, since the heart is especially vulnerable to anthracycline-mediated toxicity (Chatterjee et al., 2009). Additionally, CR + 50 mg/kg/day resveratrol also attenuated doxorubicin-mediated increases in serum LDH levels, the extracellular appearance of which is considered a general marker of tissue damage (Brancaccio et al., 2010; Glick, 1969). Importantly, we could not observe any morphological changes usually associated with doxorubicin administration (such as cytoplasmic vacuolization and myocardial fibrosis), in the left ventricle of animals treated with doxorubicin. However, these changes have usually been reported in models of

chronic doxorubicin administration over a period of few weeks (Chatterjee et al., 2009; Rahman et al., 1982; Working et al., 1999; Yi et al., 2006), in contrast to the acute doxorubicin toxicity model used in the present study. Importantly, a previous study has shown that a 35 % CR can be protective against doxorubicin cardiotoxicity in male Sprague-dawley rats (Mitra et al., 2007) which is in contrast to our study where no protective effect could be observed by CR alone. This could be attributed to the relatively mild CR regimen used in our study.

Finally, it is worth noting that our moderate, late-age onset CR is similar to most clinical trials of dietary restriction in humans (Racette et al., 2006; Willcox et al., 2004), where a high severity CR can be challenging and potentially deleterious. For example, a 36-year follow up study showed that mortality rate in healthy non-smoking Japanese-American men was increased when 50% CR was practiced and that a moderate 15% CR showed a trend for lower mortality (Willcox et al., 2004). In another study, a 12-month 20% CR has been shown to improve glucose tolerance (Weiss et al., 2006) and reduce DNA and RNA oxidation in the white blood cells of healthy normal and overweight person aged 50 - 60 years (Hofer et al., 2008). Studies have shown that such low intensity dietary restriction can also result in significant improvements in traditional cardiovascular risk factors such as blood pressure, blood glucose, circulating lipids and body composition, in both overweight (Racette et al., 2006; Sung et al., 2010; Wing & Jeffery, 1995) and lean individuals (Fontana et al., 2008; Meyer et al., 2006). With respect to resveratrol, although animal studies are plenty, human clinical trials investigating the cardiovascular effects of resveratrol have been comparatively limited and such trials have mostly investigated the pharmacodynamics and safety issues

associated with the drug (Smoliga et al., 2011). However, at least one clinical trial in obese men has shown that short-term (30 days) supplementation with 150 mg/kg/day resveratrol increased AMPK and SIRT1 levels in muscles and led to metabolic changes in blood similar to that of CR (Timmers et al., 2011). In conclusion, both late age-onset, mild CR regimen and the plant polyphenol resveratrol have shown numerous cardioprotective effects in experimental animal models and in controlled human clinical trials. It therefore follows that combining the two interventions would only offer additional cardioprotective effects. Although their exact mechanism of action is still a matter of debate, our results suggest that an induction of myocardial autophagy is at least partially responsible for the beneficial effects.

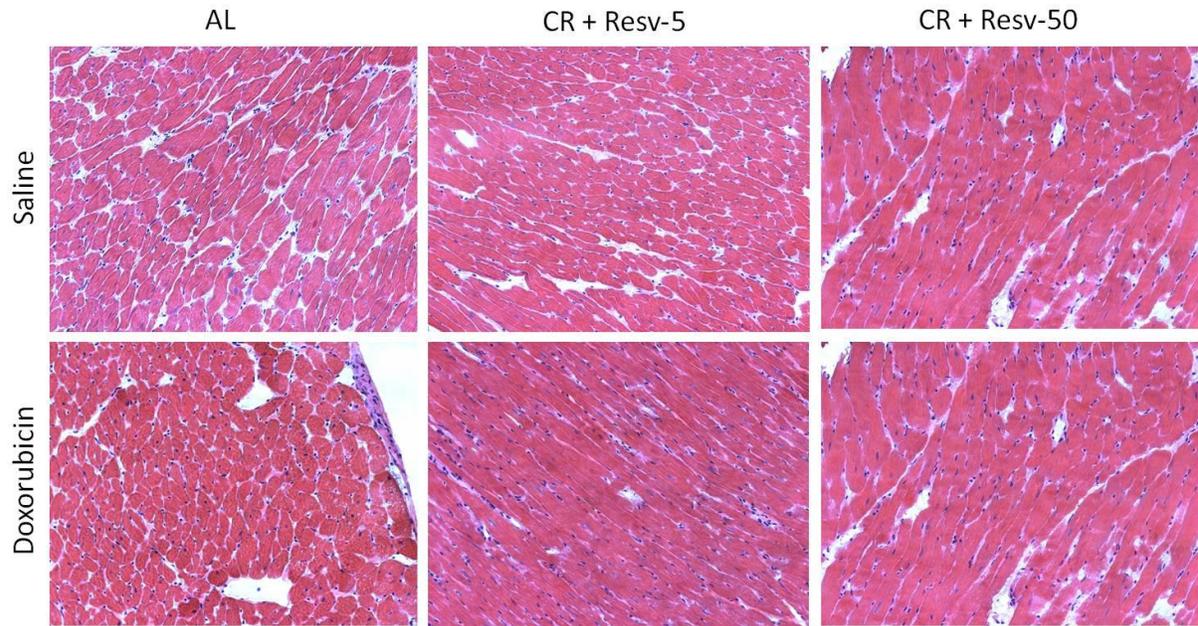


Figure 3-1. CR + Resv-5 and CR + Resv-50 interventions for 6 weeks or doxorubicin administration for 24h does not change heart morphology. Hematoxylin and eosin (H&E) histological staining was performed on 10 μ m thick sections of the left ventricular tissue isolated from saline and doxorubicin-treated AL, CR + Resv-5 and CR + Resv-50 animals. $n = 2$ to 3. Representative images are shown.

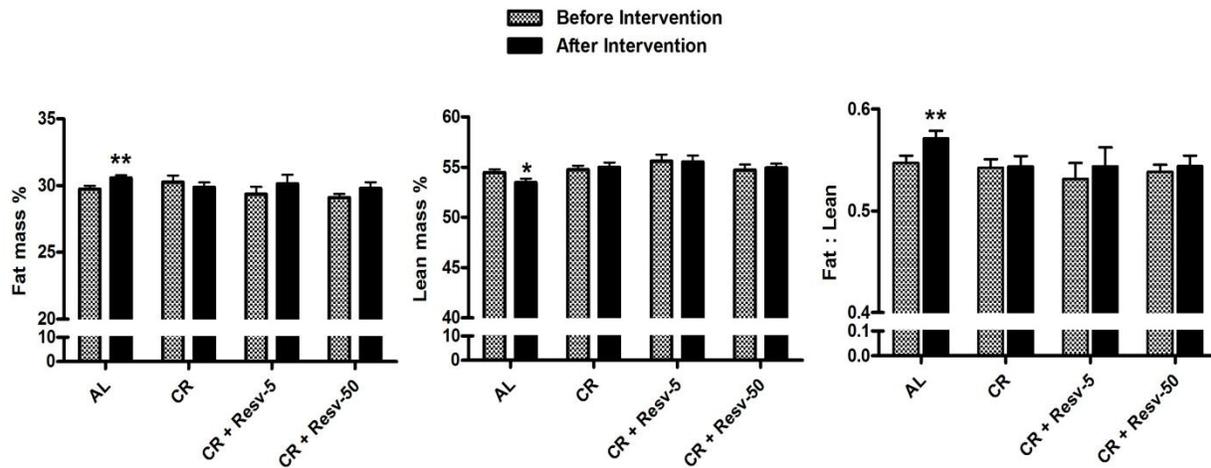


Figure 3-2. CR diet alone or in combination with resveratrol, attenuates body composition changes over time. (A-C) Fat mass %, lean mass % and Fat / Lean ratio was measured using TD-NMR analysis at baseline levels and at the end of the 6-week intervention period in AL, CR, CR + Resv-5 and CR + Resv-50 animals. AL diet significantly increased fat mass % (A), decreased lean mass % (B) and increased fat / lean ratio (C) over the 6-week intervention period. CR, CR + Resv-5 and CR + Resv-50 did not show any significant changes in body composition before and after intervention. * $p < 0.05$ vs. before intervention; ** $p < 0.01$ vs. before intervention. $n = 8$ to 10.

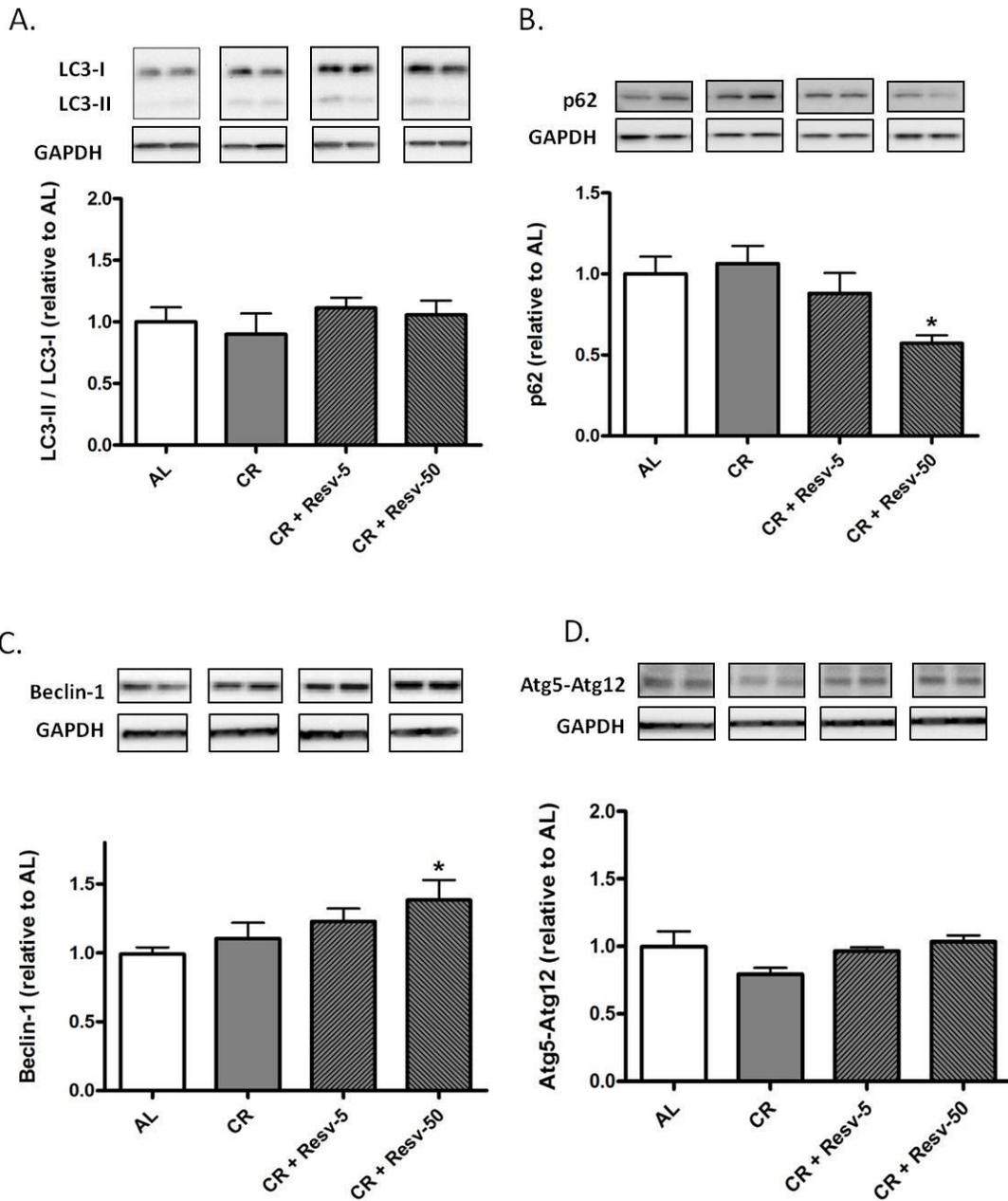


Figure 3-3. CR + Resv-50 decreases p62 abundance and increases Beclin-1 levels in left ventricular homogenates. (A-D) Protein expression levels of autophagy markers LC3B (A), p62 (B), Beclin-1 (C) and Atg5-Atg12 conjugate (D) were measured by immunoblotting in the left ventricular homogenates of saline-treated AL, CR, CR + Resv-5 and CR + Resv-50 animals. GAPDH is used as a loading control. * $p < 0.05$ vs. AL. $n = 4$ to 12.

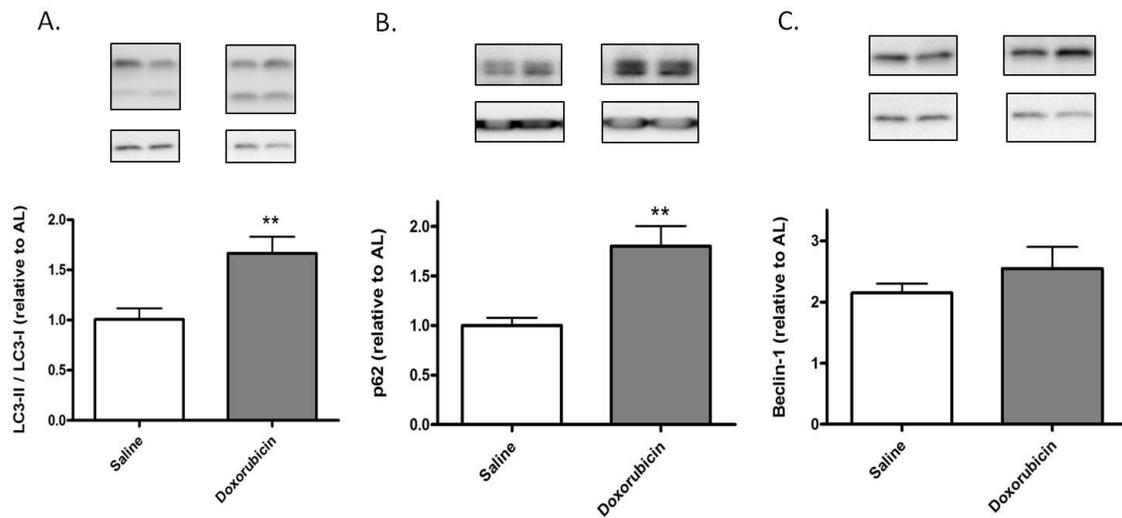


Figure 3-4. Doxorubicin increases LC3-II/LC3-I ratio and p62 accumulation in left ventricular homogenates. (A-C) Protein expression levels of autophagy markers LC3B (A), p62 (B) and Beclin-1 (C) were measured by immunoblotting in the left ventricular homogenates of saline or doxorubicin-treated animals. GAPDH is used as a loading control. ** $p < 0.01$ vs. saline. $n = 11$ to 12.

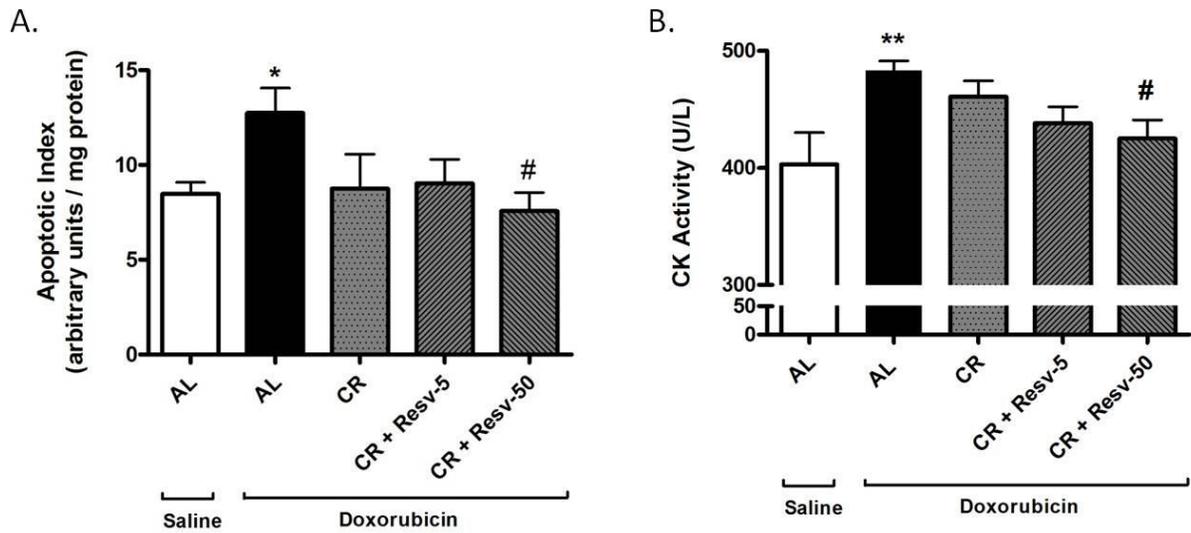


Figure 3-5. CR + Resv-50 attenuates doxorubicin-mediated increases in cardiac damage markers. (A) Apoptotic induction in left ventricular homogenates of saline-treated AL rats and doxorubicin-treated AL, CR, CR + Resv-5 and CR + Resv-50 groups was determined by measuring the release of mono and oligo- nucleosomes in the cytosolic fraction. Results have been normalized to the total amount of protein used for the assay. * $p < 0.05$ vs. AL + saline; # $p < 0.05$ vs. AL + doxorubicin (B) Serum CK levels were measured in the same groups as in (A). ** $p < 0.01$ vs. AL + saline, # $p < 0.05$ vs. AL + doxorubicin. $n = 5$ to 12.

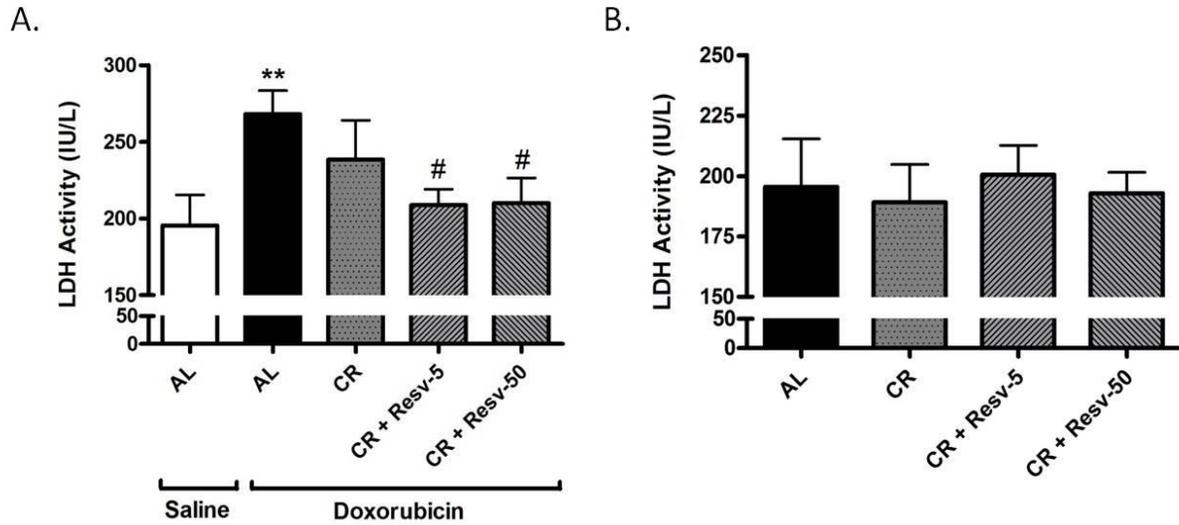


Figure 3-6. CR + Resv-50 attenuates doxorubicin-mediated increases in serum LDH activity. (A) Serum LDH activity was determined in saline-treated AL and in doxorubicin-treated AL, CR, CR + Resv-5 and CR + Resv-50 animals. ** $p < 0.01$ vs. AL + saline; # $p < 0.05$ vs. AL + doxorubicin. $n = 5$ to 12. (B) (A) Serum LDH activity was determined in saline-treated AL, CR, CR + Resv-5 and CR + Resv-50 animals. $n = 4$ to 12.

Table 3-1. Ingredient composition for AL and 20% CR purified diet.

Composition	AL	20% CR
Casein	140.00	175.00
Maltose Dextrin	230.00	230.00
Soybean Oil	40.00	50.00
Corn Starch	160.70	93.37
Cellulose	40.00	50.00
Sucrose	100.00	100.00
Dextrose	240.00	240.00
Salt mix, AIN-93MMX	35.00	43.75
Vitamin Mix, AIN-93	10.00	12.50
L-Cystine	1.80	2.25
Choline Bitartrate	2.50	3.13
Bacon Flavoring		As needed
Flowing Agents		As needed

Table 3-2. List of reagents used for in vivo study.

Reagent Name	Source	Catalog No.
Bradford Reagent	BioRad, Hercules, CA	500-0205
Cell Death Detection Plus ELISA kit	Roche Molecular Diagnostics, Indianapolis, IN	1-774-425
Dithiothreitol	Sigma, St. Louis, MO	D9779
ECL Plus reagent	Amersham Biosciences, Buckinghamshire, UK	RPN2132
EDTA	Fisher Scientific, San Jose, CA	BP120-500
Enzyhrom Creatine Kinase Assay kit	Bioassay Systems, Hayward, CA	ECPK - 100
Halt's protease inhibitor	Thermo Scientific, Fair Lawn, NJ	78439
HEPES	Sigma, St. Louis, MO	H3375
PVDF (polyvinylidene difluoride) membrane	BioRad, Hercules, CA	162-0177
Quantichrom Lactate Dehydrogenase kit	Bioassay Systems, Hayward, CA	DLDH - 100
Resveratrol	Sigma, St. Louis, MO	R5010
Sodium orthovanadate (Na ₃ VO ₄)	Sigma, St. Louis, MO	S6508
Starting Block	Thermo Scientific, Fair Lawn, NJ	37543
Tris-HCl polyacrylamide gels	BioRad, Hercules, CA	
Triton X-100	Sigma, St. Louis, MO	T9284
β-glycerophosphate	Sigma, St. Louis, MO	G9422

Table 3-3. List of primary antibodies used for in vivo study.

Species	Antigen	Source	Catalog No.
mouse	anti-Atg5	Sigma, St. Louis, MO	A2859
rabbit	anti-Beclin-1	Cell Signaling Technology, Danvers, MA	3738
rabbit	anti-LC3B	Cell Signaling Technology, Danvers, MA	2775
rabbit	anti-p62	Cell Signaling Technology, Danvers, MA	5114
mouse	GAPDH-HRP	Sigma, St. Louis, MO	G9295

Table 3-4. Body weight (BW) and heart weight (HW) analysis.

	AL	CR	CR + Resv-5	CR + Resv-50
BW (g)	581 ± 39	529 ± 45**	519 ± 31***	521 ± 35***
HW (g)	1.36 ± 0.08	1.33 ± 0.13	1.25 ± 0.06***	1.24 ± 0.09***
HW/BW	2.34 ± 0.17	2.52 ± 0.18	2.43 ± 0.22	2.38 ± 0.16
RA (g)	0.041 ± 0.011	0.060 ± 0.028	0.050 ± 0.060	0.036 ± 0.009
RV (g)	0.205 ± 0.029	0.216 ± 0.029	0.187 ± 0.026	0.187 ± 0.034
LA (g)	0.040 ± 0.009	0.044 ± 0.019	0.034 ± 0.007	0.035 ± 0.007
LV (g)	0.523 ± 0.068	0.555 ± 0.053	0.505 ± 0.079	0.477 ± 0.058

RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle. **p < 0.01, ***p < 0.001 vs. AL. n = 9 to 23.

Table 3-5. Plasma resveratrol concentrations analysis.

Intervention groups	Resveratrol concentration (ng/mL)
AL	ND
20% CR + Resv-5	7.4 ± 3.7
20% CR + Resv-50	41 ± 19

ND, not detectable. $n = 5$ in each group.

Table 3-6. Blood glucose analysis in saline and doxorubicin-treated rats

	Saline (mg/dl)	Doxorubicin (mg/dl)
AL	112 ± 17	132 ± 33
CR	103 ± 10	129 ± 24
CR + Resv-5	103 ± 10	124 ± 17
CR + Resv-50	102 ± 11	118 ± 30

n = 4 to 12.

CHAPTER 4

CONCLUSION

Summary of Results and Therapeutic Relevance

Age-related increases in oxidative damage have been well documented in the heart and is implicated in age-associated decline of cardiac function (Marzetti et al., 2009). Oxidative stress can damage cellular organelles, especially the mitochondria, which further enhances oxidative burden by generating more ROS. Under such circumstances, the removal of oxidatively damaged components and dysfunctional mitochondria from cells would be highly desirable and potentially beneficial. This holds true especially for cardiomyocytes, due to their post-mitotic nature and a lifespan of decades. Autophagy serves this essential homeostatic clean-up function. The relevance of the autophagy-lysosomal axis to cardiomyocyte homeostasis is witnessed by the observation that abnormal oxidant generation, mitochondrial dysfunction and the accumulation of intracellular waste material are observed under conditions of inhibited autophagy (Nakai et al., 2007b). This suggests that the optimization of the housekeeping function of autophagy may be harnessed as a therapeutic means against oxidative damage and heart senescence. We hypothesized that enhancement of basal autophagy in cardiomyocytes will protect against pathological consequences resulting from enhanced generation of oxidative stress. We tested this hypothesis both in vitro (HL-1 cardiomyocytes) and in vivo (26-month-old rat hearts).

For our in vitro study (Chapter 2), we investigated in detail, the role of mitochondria-specific ROS generation in mediating cellular cytotoxicity and whether autophagy enhancement could be protective under such circumstances. We observed that treatment of HL-1 cardiomyocytes with the antibiotic compound AMA increased $O_2^{\bullet-}$

generation, decreased $\Delta\psi_m$ and inhibited cellular respiration (Fig. 2-1). In addition, AMA augmented nuclear DNA oxidation and cell death (Fig. 2-1). Although oxidative stress per se has been reported to be a potential inducer of autophagy, we did not observe autophagic stimulation by AMA treatment (Fig. 2-2). Induction of autophagy and mitophagy by the mTOR inhibitor rapamycin protected cardiomyocytes from the cytotoxic effects of AMA, as assessed by viability assays and PARP-1 and caspase-3 activation analysis (Fig. 2-5). In addition to an overall effect on cell survival, rapamycin-mediated autophagy improved mitochondrial function, as determined by respiration analysis, $\Delta\psi_m$ and mitochondrial morphology analysis (Fig. 2-7).

In addition to improving mitochondrial function, rapamycin suppressed the accumulation of ubiquitinated proteins induced by AMA (Fig. 2-6). The ubiquitinated proteins were observed only in the detergent insoluble fraction of AMA-treated cells, indicating that these proteins could be aggregated in nature. Autophagy induction by rapamycin was able to effectively mediate their clearance (Fig. 2-6). This observation is consistent with previous studies illustrating a role of autophagy in removing protein aggregates in cell culture models of aggregate-prone diseases (Harada et al., 2008; Ravikumar et al., 2004).

In Chapter 3, we have summarized our findings from in vivo studies investigating interventions targeted to induce autophagy in 26-month-old FBN rat hearts. Studies investigating autophagic stimulation in late-middle-aged or old animals are very limited and often the interventions proposed (such as lifelong 40% CR) are unfeasible in humans. We have tested a moderate CR regimen (20% CR) alone, or in combination with the plant polyphenol resveratrol (oral dosing) starting late in life and for a period of

6 weeks, as potential inducers of autophagy. Analysis of autophagy markers in the left ventricle of heart tissue revealed that 20% CR in combination with 50 mg/kg/day resveratrol can decrease the abundance of p62 (indicating enhanced autophagic flux) (Fig. 3-3) and enhance the expression of autophagy protein Beclin-1 (indicating a possible induction of autophagy) (Fig. 3-3). Our observation that a moderate CR regimen and resveratrol, only when combined, stimulated autophagy indicates a potential synergistic action between the two. The exact mechanism through which the combined approach induces autophagy is currently unknown and can only be speculated. Both CR and resveratrol have been shown to inhibit the mTOR pathway and activate AMPK and SIRT1, all of which can induce autophagy through independent ways (Fig. 4-1).

Furthermore, similar to *in vitro* studies, we investigated whether enhancing basal levels of cardiac autophagy can be protective against oxidative stress-mediated damage. Oxidative stress was generated in FBN rats by intraperitoneal administration of doxorubicin, a widely used chemotherapeutic agent, reported to cause significant cardiotoxicity. Our group has previously shown that doxorubicin increases cardiac levels of 8-iso prostaglandin $F_{2\alpha}$, a known marker of lipid peroxidation (Childs et al., 2002). In addition, it enhances cytochrome *c* release, caspase-3 activation and apoptotic levels and in the myocardium (Childs et al., 2002). We observed that the induction of autophagy by the CR + resveratrol regimen attenuated doxorubicin-mediated increases in cardiac apoptotic levels (Fig. 3-5), serum CK (Fig. 3-5) and LDH levels (Fig. 3-5), supporting the conclusion that enhanced autophagy serves to protect the myocardium from doxorubicin-mediated damage. It is worth noting here that doxorubicin is mostly

administered to patients in late-middle-ages, at which stage the incidence of cancer is among the highest. Our study was conducted in 26-month-old FBN rats which correspond to about 60 years (late-middle-age) in humans (Quinn, 2005). Hence, the study holds clinical and translational relevance.

Our study conclusively shows that the upregulation of autophagy is protective against oxidative stress-mediated dysfunction in cardiomyocytes. Investigations into the mechanisms of such a protective effect showed that at least in cell culture models, autophagy induction leads to the clearance of aggregated, ubiquitinated proteins and dysfunctional mitochondria. The protective effect of autophagy induction was also seen in an animal model of oxidative stress-mediated injury, where autophagy was induced by nutritional interventions such as CR and resveratrol. A mild CR regimen starting late in life and the use of plant polyphenol resveratrol are feasible interventions that could be used in humans. The 50 mg/kg/day oral dose of resveratrol used in the study (in combination with CR) translates to a dose of approximately 4 mg/kg/day resveratrol in humans (Reagan-Shaw et al., 2008). In other words, a human weighing 60 kg would need a daily oral dose of 240 mg resveratrol. This dose of resveratrol is much lower than what has been reported to cause side-effects in humans (Patel et al., 2011). No clinical trial has investigated a combinatorial approach of a moderate CR regimen with resveratrol supplementation, and therefore such a study would be highly desirable. Until then, we propose that following a lifestyle of consuming fewer calories, combined with a daily oral consumption of resveratrol may have potentially beneficial effects in humans.

Our in vitro and in vivo studies therefore illustrate the crucial role of autophagy in attenuating cardiac oxidative damage. Others have demonstrated the contribution of

autophagy in preserving cardiomyocyte physiology in general (Nakai et al., 2007b). With the beneficial roles of autophagy being increasingly reported, a lot of attention has also been focused on identifying novel enhancers of autophagy. Autophagy inducers can be broadly categorized to be acting through mTOR-dependent and -independent pathways. Rapamycin and its homologs comprise mTOR-dependent inducers of autophagy, which act by inhibiting mTOR, thereby removing its inhibitory effect on autophagy induction (Ganley et al., 2009; Jung et al., 2009a). In collaboration with other investigators at the University of Florida, we have observed that feeding orally 2.24 mg/kg/day rapamycin for 8-10 months can enhance autophagy in the hearts of C57BL/J6 mice (Fig. 4-2). Rapamycin used in the study was encased using an acrylic polymer spinning disk enteric coating system to prevent digestion in the stomach and thereby increase the bioavailability of rapamycin. We observed increased levels of LC3-II/LC3-I ratio (Fig. 4-2A) in whole heart homogenates from these mice, along with decreases in the abundance of p62 (Fig. 4-2B). No changes were observed in the protein expression levels of Beclin-1 (Fig. 4-2C). Such observations complement our in vitro finding where rapamycin significantly induced autophagy in mouse HL-1 and human AC16 cells. Other researchers have shown that induction of autophagy by rapamycin removes mutant huntingtin aggregates and offers protective effects in cell culture systems and in transgenic *Drosophila* and mouse models of Huntington disease (Berger et al., 2006; Ravikumar et al., 2002; Ravikumar et al., 2004). Apart from rapamycin, a recent screening of a library of 3500 chemicals has identified four compounds: perhexiline, niclosamide, amiodarone and rottlerin, which significantly induced autophagy through the mTOR pathway (Balgi et al., 2009). Among these chemicals, rottlerin inhibited

mTORC1 by activating TSC2, while the rest acted in a TSC2-independent manner (Balgi et al., 2009). Importantly, perhexiline (used for treating chest pain arising from an ischemic heart), niclosamide (anti-helminthic agent) and amiodarone (used against cardiac arrhythmias) are also FDA approved drugs.

The very first evidence of mTOR-independent induction of autophagy came from the observation that a decrease in cellular levels of inositol 1,4,5-triphosphate (IP₃) can stimulate autophagy, with no effect on the mTORC1 or mTORC2 pathway (Sarkar et al., 2005). IP₃-mediated regulation of autophagy is most likely to be due to IP₃ being required for ER Ca²⁺ release. For instance, increased levels of cytosolic IP₃ can bind to IP₃ receptors (IP₃R) in the ER and trigger the release of ER-sequestered Ca²⁺ in the cytoplasm (Berridge, 1993). Increased cytosolic calcium can inhibit Ca²⁺ dependent cysteine proteases called calpains and this leads to an inhibition of autophagy (Demarchi et al., 2007; Demarchi et al., 2006; Gordon et al., 1993). Accordingly, lithium and other mood stabilizing agents (such as carbamazepine and sodium valproate), which reduces intracellular inositol levels by inhibiting inositol monophosphatase, enhanced autophagy and mediated the clearance of autophagy substrates (Berridge, 1993; Sarkar et al., 2005; Williams et al., 2002). Similarly, inhibition of IP₃R by xestospongine B also stimulated autophagy (Criollo et al., 2007). In another remarkable study, a high-throughput screening of 50,729 compounds resulted in the identification of three small molecule enhancers of autophagy, which increases autophagosome formation and stimulates the clearance of autophagy substrates (Sarkar et al., 2007). The molecules were found to act in an mTOR-independent manner, although the precise cellular mechanism is yet to be known (Sarkar et al., 2007).

In spite of the identification of numerous autophagy inducers, it is important to note that the majority of these experiments were conducted in cell culture models. Whether the abovementioned compounds could also stimulate autophagy in vivo remains to be established. Measuring autophagic flux in animal models is limited by the fact that lysosomal inhibitors cannot be directly used in vivo. Although at least one paper reported the use of chloroquine to inhibit lysosomal degradation in animals (Iwai-Kanai et al., 2008), follow-up studies have not been conducted. The inhibition of lysosomal degradation in vivo therefore remains extremely difficult and rare. Autophagy flux determinations in animals can partly be accomplished by indirect methods such as by analyzing p62 abundance or by measuring the clearance of mutant, aggregate-prone proteins in mouse models of huntingin (Lin et al., 2001; White et al., 1997) or alpha-synuclein aggregation (Chesselet, 2008).

Finally, while the self clean-up process of autophagy is critical for the maintenance of cell homeostasis, nevertheless, an excessive activation of autophagy may be maladaptive, chewing up essential survival components and leading to further toxicity. Increased number of autophagic vacuoles have been observed under pathological conditions, such as in the cardiac tissues of patients with heart failure (Kostin et al., 2003), aortic valve stenosis (Hein et al., 2003), left ventricular hypertrophy (Yamamoto et al., 2000) and the hibernating myocardium (Elsasser et al., 2004). However, it is unclear whether these autophagic vacuoles were actually contributing to the pathological conditions or whether it was upregulated in an attempt to protect against cell death. For example, up-regulation of autophagy might represent an attempt to cope with increased levels of mitochondrial dysfunction and cellular damage. Nevertheless, a

detrimental aspect of excessive autophagic stimulation has not been conclusively ruled out. The level of autophagy may be critical in determining whether autophagy will be protective or detrimental. Therapeutic interventions that exploit the homeostatic properties of autophagy, without stimulating its maladaptive effects caused by an excessive induction, is therefore highly desirable.

Future Directions

Although the induction of basal autophagy protects cardiomyocytes from enhanced oxidative damage and despite the recent evidences supporting the involvement of impaired autophagy in the accumulation of oxidative damage within old cardiomyocytes, several research questions still remains to be addressed. First, whether enhancing autophagy can attenuate cardiac senescence needs to be clearly established. In this regard, Harrison and co-authors reported that oral administration of rapamycin to mice, starting at 600 days of age, extended median lifespan in male and female mice by 9% and 14% respectively (Harrison et al., 2009). Although the precise molecular mechanisms leading to such an effect was not investigated in the study, it can be hypothesized that autophagy induction was at least partially responsible for such an effect. Additionally, studies investigating whether manipulation of autophagy rescues the premature heart senescence phenotype observed in animal models characterized by high loads of mtDNA mutation (and oxidative stress) would be highly desirable. Second, autophagy is not just a random degradation process; rather, it is highly regulated and potentially selective. Therefore, it is mandatory to understand whether specific signal transduction pathways or the engulfment of particular autophagy substrates are negatively affected under conditions of increased oxidative stress or during senescence. With regard to the mitochondria, the identification of signaling

pathways linking mitochondrial dynamics and selective mitophagy is necessary for the development of therapeutics that maximize the removal of damaged mitochondria, while sparing functional ones. Third, it should be considered that most autophagy mediators have multiple functions, meaning that their manipulation may produce unrelated and perhaps undesirable effects. Therefore, a deeper understanding of the various actions performed by autophagy-inducing compounds / interventions is warranted. Fourth, whether the optimization of autophagy preserves cardiomyocyte mitochondrial function and delays the development of CVD in humans remains to be established. Most data on the effects of pharmacological or behavioral modulation of autophagy on cardiac aging and physiology derive from model organisms in which the role of autophagy may differ from what impacts human health, partly because of difficulties in modeling complex human diseases and degenerative processes in experimental settings.

The current limitations make extremely challenging, the identification of specific autophagy inducers and also the optimal window of autophagic activation to exploit the cardioprotective effects of autophagy without any non-specific, undesirable or potentially detrimental effects. Answering the above critical research questions will likely provide cardiologists and geriatricians with novel therapeutic means to postpone the degenerative fate of cardiomyocytes through the induction of autophagy and relieve the burden associated with increased oxidative stress common at old age and in CVD patients. In summary, the vital functions carried out by the autophagic process in cardiac physiology suggest the possibility that therapeutic interventions targeting this cellular pathway may represent effective means to counter heart senescence and age-related damages due to oxidative stress. Untangling the complexity of autophagic

regulation and managing the dual nature of autophagy are major tasks the field of geriatric cardiology is called to pursue.

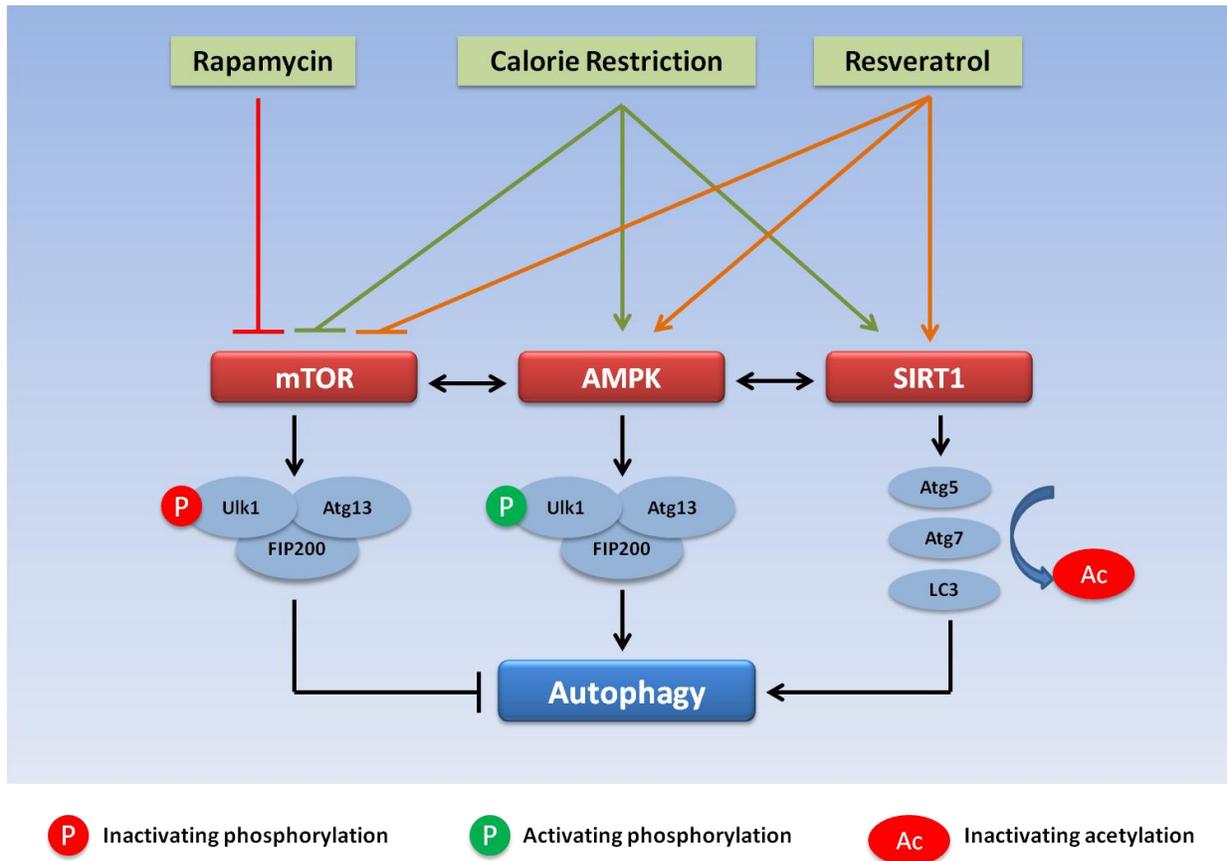


Figure 4-1. Regulation of autophagy by rapamycin, CR and resveratrol. Rapamycin inhibits mTOR kinase and thereby removes mTOR-dependent inhibitory phosphorylation on the ULK1 – Atg13 – FIP200 complex. Removal of inhibitory phosphorylation results in the induction of autophagy. Both CR and resveratrol can activate autophagy by inhibiting the mTOR pathway and/or by activating of the AMPK and SIRT1 pathways. AMPK induces activating phosphorylation on the ULK1 – Atg13 – FIP200 complex, thereby stimulating autophagy. SIRT1 can decetylate and activate key autophagy genes Atg5, Atg7 and LC3, thereby inducing autophagy.

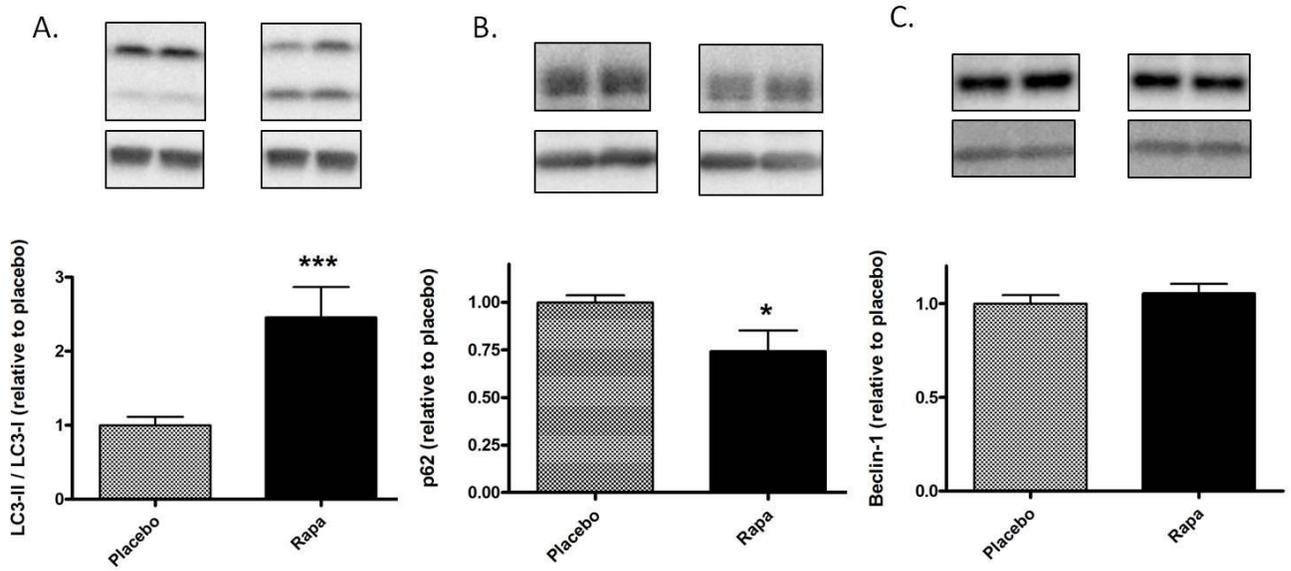


Figure 4-2. Chronic rapamycin administration induces autophagy in C57BL/J6 mice hearts. Protein expression levels of autophagy markers LC3B (A), p62 (B) and Beclin-1 (C) were measured by immunoblotting in whole heart homogenates of placebo or rapamycin-treated animals. Rapa, Rapamycin. * $p < 0.05$, *** $p < 0.001$ vs. placebo.

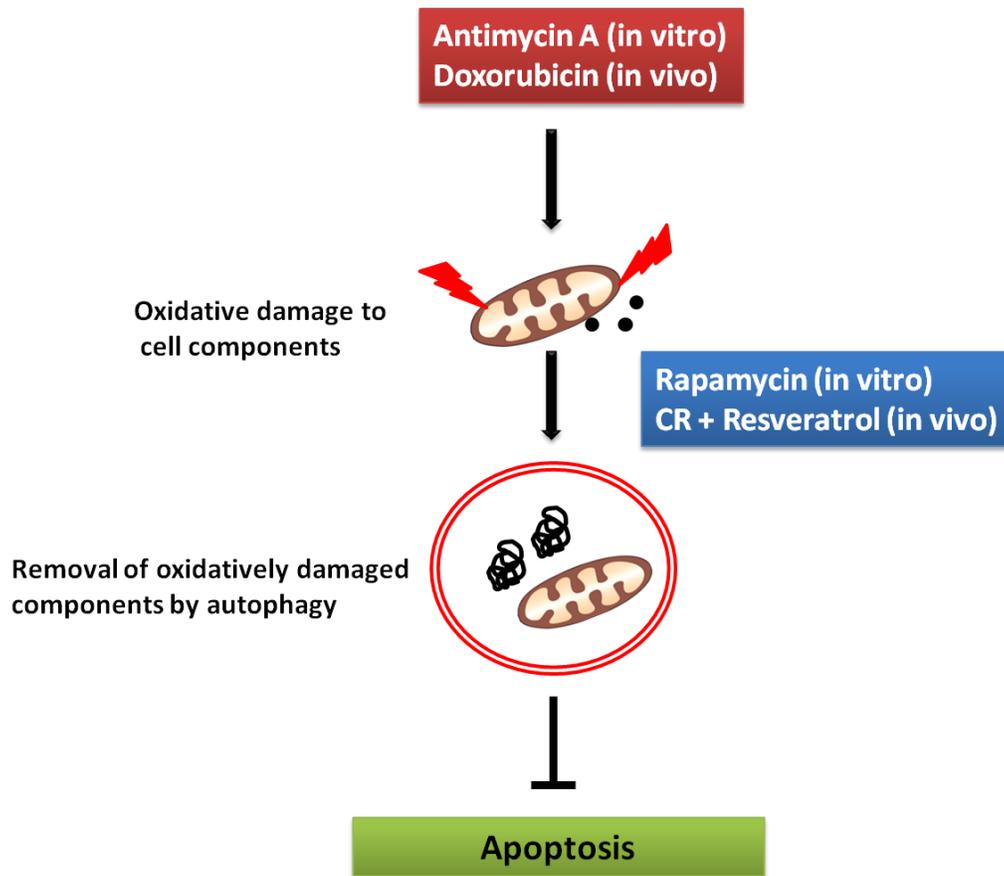


Figure 4-3. Schematic of overall observations from in vitro and in vivo studies. Oxidative stressors such as AMA and doxorubicin can cause damage to cellular components, affecting cell viability. Induction of autophagy by rapamycin in vitro (HL-1 cells) and by a combined intervention of CR and resveratrol in vivo (FBN rat hearts) can improve cell survival and homeostasis by potentially removing oxidatively modified components.

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BIOGRAPHICAL SKETCH

Debapriya was born in Howrah, India, to Pradip Narayan Dutta and Kanika Dutta. She obtained her primary education from St. Thomas High School, Howrah, and then from Shri Shikshayatan, Kolkata. After completing high school, Debapriya joined Bengal College of Engineering and Technology, affiliated to West Bengal University of Technology (WBUT), India, to pursue a Bachelor of Engineering degree in Biotechnology. She graduated from WBUT in 2005 and after completing an internship at Rajabazar Science College (affiliated to Calcutta University, India), Debapriya joined the Interdisciplinary Program (IDP) in Biomedical Sciences at the University of Florida in 2006. Debapriya joined Dr. Christiaan Leeuwenburgh's lab in 2008, and was co-mentored with Dr. William A. Dunn, Jr. Her research was primarily focused on investigating the role of autophagy in alleviating oxidative stress-induced damage in cardiomyocytes. Debapriya graduated in summer, 2012.