p53 Mediates Cell Death in the Heart

by

Zeinab Roveimiab

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Department of Physiology & Pathophysiology

Max Rady College of Medicine

Rady Faculty of Health Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

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Abstract

During cellular stress, cells try to survive and overcome the stress by an essential process called autophagy. This process involves degrading and recycling organelles and macromolecules. Different human diseases such as heart failure are on the rise due to changes in our diet and these diseases can be worsened by defects in the regulation of this very important process, autophagy.

Doxorubicin as an antitumor drug induces tumor suppressor protein p53 activation in the heart, which causes mitochondria defects, autophagy and cell death in cardiomyocytes. Also, in this thesis, I found that overexpression of p53 increases the level of the autophagy marker, Beclin 1, that is activated in the autophagy's first stage, which is the autophagosome formation, by making a double membrane structure that engulfs cytoplasmic material.

These data provide evidence that Doxorubicin induces p53 which activates autophagy and cardiomyocytes cell death. Therefore, the results of this thesis demonstrate that when p53 is activated, it can promote cardiomyocyte cell death through an autophagy dependent process during cardiac stress.

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To my husband, Kian
for his love, patience, and understanding;

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for their lifetime devotion;

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for their constant encouragements.

List of Abbreviations

Atg - autophagy related genes

Bcl 2 - B-cell lymphoma 2

Bnip3- Bcl2 family 19-KDa interacting protein

Cyto C- cytochrome C

DMEM - Dulbecco's Modified Eagle's Medium

DRP1- dynamin-related protein 1

FBS - fetal bovine serum

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

HPX - hypoxia

IMM- inner mitochondrial membrane

I/R - ischemia/reperfusion

LC3- microtubule light chain 3

MFN - mitofusin

mPTP- mitochondrial permeability transition pore

mTOR - mammalian target of rapamycin

OMM- outer mitochondrial membrane

PBS - phosphate buffered saline

PI3K III - type III PI3 kinase

p53- tumor suppressor protein encoded by the TP53 gene

RIP- receptor interacting protein

ROS - reactive oxygen species

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

TMRM- tetramethylrhodamine, methyl ester

$\Delta\psi m$ - mitochondrial membrane potential

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1. Introduction

Heart failure is a chronic disease, which results in the heart being unable to pump blood sufficiently to meet the demands of the body. It is the leading cause of death worldwide as population of elderly people is on the rise [1]. The main cause of heart failure is loss of cardiomyocytes. Adult cardiac myocytes lose the ability of dividing shortly after birth [2]. We are born with a limited number of cardiac myocytes and they do not have the ability to proliferate [2]. Therefore, the number of cells does not increase and reduction in cells will reduce cardiac function and thereby cause cardiovascular diseases such as heart failure.

There are three types of cell death: apoptosis, necrosis, and autophagy. Apoptosis is a form of programmed death that requires energy (ATP dependent) and activation of caspase proteolytic cascades. Necrosis was initially known as unprogrammed cell death. However, growing evidence suggests that necrosis can also be regulated and be part of the inflammatory pathway that leads to cell death. Autophagy means self-eating and involves lysosomal mediated degradation of cytoplasmic structures to recycle nutrient and energy during times of cell crisis. The removal of damaged organelles typically occurs by autophagy, which is important for maintaining cellular homeostasis. Too much or too little autophagy is harmful and causes cell death and disease. In all types of heart disease, autophagy becomes activated, and in some cases is adaptive. However, in some cases, it can be maladaptive and leads to progression of the disease.

Doxorubicin (DOX) as a chemotherapeutic agent is used for treating the most of human cancers worldwide such as lung, breast and lymphoma cancers. DOX has cardiotoxic effects (acute and chronic) such as causing defects in mitochondria, death of cardiomyocytes, reduced heart function and finally leading to heart failure [37]. DOX's cardiotoxicity is not been fully known.

DOX induces tumor suppressor protein p53 in the heart, which causes mitochondria defects, autophagy and cell death in cardiomyocytes.

The p53 tumor suppressor protein arrests cell cycles, causes death in damaged cells, and prevents proliferation in cancer cells [18]. The level of p53 is low under normal conditions and rises with stress crisis such as DNA damage [21]. Mitochondria has a vital role in regulating cellular respiration and energy metabolism. p53 causes mitochondrial defect, and a shift in metabolism of cardiomyocytes from oxidative metabolism to glycolysis to overcome crisis.

p53 causes cell death in cardiac myocytes by mitochondria dependent autophagy. Pervious data from our laboratory have revealed that p53 localizes to the mitochondria and perturbs their function by loss of $\Delta\psi m$ and mPTP opening. Finally, the removal of damaged mitochondria is by an autophagic process.

The data presented in this thesis supports the idea that p53 provokes mitochondrial injury, leading to autophagic cell death in ventricular cardiomyocytes. However, the mechanism by which p53 causes mitochondria injury and autophagy in cardiac cells is not fully understood. Knowledge regarding the p53 protein and maladaptive autophagy will be beneficial to cardiac biology and to find a new therapeutic regime for cardiovascular diseases.

2. Literature Review

2.1. Heart Failure

Heart is a muscular and important organ of the body that works as a pump to supply the whole body's organs and tissues with nutrient and oxygen continuously through circulatory system and removes wastes and carbon dioxide. There are two types of circulation, pulmonary and systemic. The heart receives oxygenated blood from the lung through the pulmonary circulation. The oxygenated blood goes to the whole body from heart through the systemic circulation and comes back as the deoxygenated blood to the heart to be sent to the lung to be oxygenated.

Heart failure (HF), a chronic disease, makes heart unable to pump blood sufficiently through the body to satisfy oxygen and nutrient demands of organs and tissues. The main symptoms are shortness of breath, swelling in leg and ankle, and fatigue [1]. Heart failure is a serious condition with no known cure. HF due to hypertension, myocardial infarction, and coronary artery disease is decreasing since it is being increasingly recognized and treated worldwide [2], while HF due to other risk factors such as obesity and diabetes is increasing. Also, the incidence of HF is increasing as the population of elderly people is on the rise over years. As statistics have shown between 2011 and 2014, there were approximately 6.5 million cases of HF among American adults at the age of 20 and older, while records between 2009 and 2012 showed 5.7 million cases of HF [3].

The main cause of the HF is loss of cardiomyocytes. Adult mammalian heart cells lose the ability of dividing and repairing shortly after birth. All types of cell death such as apoptosis, necrosis, and autophagy have been seen in heart diseases [4]. Fibrillar collagen replaces lost cardiomyocytes which causes heart muscle to lose its flexibility and stiffen, making it harder for the heart to contract.

Studying cell death pathways at molecular level is essential to develop therapeutic agents to prevent cell death in order to prevent the loss of cardiac function after injuries such as myocardial infarction, myocarditis, etc.

2.2. Cell Death in the Heart

The quality control mechanism for the development of multicellular organisms is cell death. Too much or too little of it will cause pathogenesis of different diseases, such as cardiovascular, neurodegenerative diseases, and cancer [5]. Cardiomyocytes do not have the ability to replicate after birth. If sufficient cell death happens due to different factors such as hypertension or ischemia, the heart fails due to inability to make new cells to repair itself. Therefore, it is very important to understand heart cell death mechanism in order to understand heart failure.

Mitochondria are important multifunctional organelle. They are the main source of energy (ATP), involved in multiple metabolic reactions, and control cell death and cell survival. The involvement of mitochondria in both vital and lethal processes is very important for maintenance of cell homeostasis. Any changes in signaling pathways in the mitochondria of cardiac muscle cells can cause cardiovascular disorders, such as ischemia injury, myocardial infarction, and HF [6].

Cardiomyocytes death can happen through three pathways: apoptosis, necrosis, and autophagy. Mitochondria, as the cellular energy source, go under apoptosis or necrosis or autophagy death in response to cellular stress such as ischemic injury and infection.

2.2.1. Apoptosis

Apoptosis is a well-known form of programmed cell death and is an important mechanism during organism development to remove damaged or unwanted cells [7]. Apoptosis is an energy required procedure (ATP dependent) which is also dependent on caspase cascades [9]. Apoptotic cell starts to bleb, after which the cell size decreases due to chromatin and cytoplasmic condensation. Then, the cell can fragment into apoptotic bodies and an intact plasma membrane encloses them. Finally, the cell fragments are engulfed and degraded by phagosomes [7]. Apoptosis does not cause inflammation in contrast to necrosis [9].

There are two types of apoptosis: extrinsic and intrinsic pathways. The extrinsic cell death pathway is known as receptor mediated apoptosis which is activated by the binding of tumor necrosis factor-α (TNFα) to the death receptor, TNFR, on the plasma membrane [7]. This binding causes the formation of a multi-protein complex, containing TRADD (TNFR with death domain; DD), FADD (FAS-associated with DD), RIP1 (receptor interacting protein 1), RIP3 (receptor interacting protein 3), and caspase-8 [8]. By auto-activation of caspase-8, other death effector caspases, such as caspase-3, -6, and -7 are activated. Also, caspase-8 causes Bid (a Bcl-2 family member) protein to attach to the outer mitochondrial membrane (OMM), and by changing mitochondrial permeability leads to apoptosis [8].

The intrinsic death pathway is known as mitochondria mediated pathway, which is activated by hypoxic conditions, UV radiation, and DNA damage [8]. Mitochondria are needed to provide cellular energy through ATP production in response to cellular stress. Bax and Bak (Bcl-2 family member) proteins [9] disturb mitochondrial membrane by attaching to the OMM and forming channels in it. This causes the membrane to become more permeable, releasing cytochrome-c into

the cytosol. Then, the apoptosome structure forms, which activates caspase-9 and caspase-3, promoting apoptotic cell death [9].

2.2.2. Necrosis

Necrosis was previously known as the sudden and unprogrammed cell death, but there is growing evidence that it is regulated and programmed like apoptosis [10]. Necrosis is activated when energy level of cell suddenly drops (for instance, during heart attack), causing the loss of the membrane potential of inner mitochondrial membrane (IMM) and resulting in the cell to swell [10]. Therefore, the outer membrane of cell disrupts and leads to the leakage of the intracellular content out to the extracellular space.

In contrast to apoptosis, which leads to OMM permeabilization, necrosis causes a pore opening in the IMM. Normally, mitochondria's IMM is not permeable to ions, proteins, and water. In the matrix of mitochondria, there is an electrons transport system alongside the respiratory chain for substrate metabolism. Protons from the matrix are pumped into the intermembrane space, which creates an electrochemical gradient ($\Delta\psi m$) between the intermembrane space and matrix and provides the potential energy necessary to drive ATP synthesis. Ca^{2+}, which triggers necrosis, causes opening of the mPTP and leads to the loss of $\Delta\psi m$ and termination of ATP synthesis in mitochondria. As the result, water passes through the matrix down its osmotic gradient, leading to mitochondrial swelling and OMM rupture [11].

In contradiction to apoptosis, necrosis is not energy (ATP) and caspase dependent. In the absence of caspase-8, RIP1 and RIP3 are phosphorylated and cause IMM permeabilization [11]. Once mPTP opens, the cell swells up and the OMM ruptures. Consequently, the whole cell content is released to the extracellular space, causing inflammation [11].

2.2.3. Autophagy

Autophagy is the process of cells' "self-eating". It is a catabolic pathway which is performed to remove damaged or excessive cellular organelles like mitochondria [12]. Autophagy involves the lysosomal mediated degradation of cytoplasmic structures to recycle nutrient and energy during periods of cellular starvation. Under normal conditions, the rate of autophagy is low. However, under situations such as starvation, stress, or ischemia, the rate of autophagy increases immediately [12]. Autophagy needs to be tightly regulated, otherwise it will lead to human diseases, such as heart failure, cancer, and neurodegenerative diseases [13].

There are three types of autophagy known. The first type is 'macroautophagy', which is referred to as autophagy and is the focus in this thesis. The other two types are 'microautophagy' and 'chaperone-mediated-autophagy'. In microautophagy, cytoplasm components move to the lysosome directly through membrane invaginations [13]. In chaperone-mediated-autophagy, proteins containing the KFERQ-like motif translocate selectively through the lysosomal membrane [14].

Cell death by autophagy is termed as Type-II programmed cell death. However, it is not dependent on the caspase cascade. The markers for autophagy are: LC3-II (microtubule light chain 3-II), p62 (62 KD protein), and Beclin 1 (from Bcl-2 family) [14]. Autophagy starts with Beclin 1 protein, which leads to the formation of a double membrane autophagosome which is sent for lysosomal degradation to recycle nutrients and energy.

2.2.3.1. Mitophagy

The removal of damaged Mitochondria by autophagy is called Mitophagy [16]. Mitophagy is required as a quality control procedure to remove the damage parts of mitochondria and can happen

once mitochondrial fission occurs [16]. Mitophagy can protect cardiomyocytes if adequate number of healthy mitochondria can provide support to meet the metabolic demand of the cells.

Mitophagy marker proteins are Parkin, PTEN induced putative kinase-1 (PINK1) and mitochondrial fusion-2 proteins (MFN2) [15]. PINK1 is constitutively expressed in mitochondria and is degraded rapidly by proteolysis. When the mitochondrial membrane is depolarized, PINK1 proteolysis is inhibited and it accumulates. PINK1 causes phosphorylation of Mfn2, then Parkin binds to Mfn2 which results in ubiquitination of the OMM. Ubiquitination of the OMM is a signal for the autophagosomal degradation [15].

2.2.3.2. Fission and Fusion

Mitochondrial fission (dividing/splitting) happens during mitosis in order to ensure that equal amounts of mitochondria are distributed into daughter cells. DRP1 (dynamin-related protein-1) is activated by phosphorylation. It is attached around the mitochondria equator and divides it into two daughter cells symmetrically or asymmetrically [17]. Fission also causes the distribution of mitochondria in the cell. With respect to the cell death, fission can isolate the damaged part of mitochondria from the healthy part of the mitochondria and promotes mitophagy of the damage region [17].

Mitochondrial fusion (joining) is started by tethering, that is the attachment of the outer membrane of two mitochondria. This is followed by the outer membrane fusion with the help of mitochondrial fusion-1 protein (MFN1) and MFN2. Finally, the inner mitochondrial membrane fusion happens with the help of optic atrophy-1 (OPa1) [17].

Mitochondrial fission and fusion are a quality control mechanism for mitochondrion removal and renewal. The inactivation of one activates the other one and vice versa. The fusion mechanism

allows mitochondria to combine their contents while the mitochondrial DNA is repaired, and an equal distribution of metabolites is ensured. The mitochondrial fission removes damaged part of mitochondria from the healthy part and prevents cell death. A range of mitochondrial tasks, such as energy production, reactive oxygen species (ROS) production, Ca^{2+} signaling, and cell death, are controlled by fission and fusion, which in turn allows heart to respond to body demands properly [18]. Therefore, fission and fusion play a vital role in cardiac myocytes; if one part of this pathway gets damaged, death of cardiac myocytes happen, which at the end, results in HF [18].

2.3. Molecular Regulation of Autophagy

Autophagy is regulated by autophagy related genes (Atgs) from autophagosome formation until lysosomal degradation [19]. Autophagy starts with induction, then double membrane autophagosome formation, and finally fusion of autophagosome with lysosome for recycling compounds into the cytoplasm or the total autophagic degradation of the cell.

Nutrition deprivation, oxidative stress, etc., can be the stimuli for induction of autophagy. Atg proteins, such as Atg5, Atg12, and Atg16 help the plasma membrane invaginate around the chosen organelle or macromolecule, which results in autophagosome formation [19]. When the autophagic vesicle is completely formed, most of Atg proteins are cleaved, and then the lysosome attaches to the autophagosome in order to degrade the components inside vesicle and recycles them back to plasma.

One of the targeted organelles for lysosomal degradation is mitochondria, the contents of which are digested by lysosomal enzymes and provide the cell with amino acids and other small molecules that can be used for ATP and macromolecules production.

One way to protect the heart against ischemic cardiac injury, drug-induced cardiomyopathy, and pressure overload hypertrophy is to find a pharmaceutical way to inhibit cell death by suppressing autophagy or genetic mutations of key regulatory factors of autophagy [19].

2.3.1. Autophagy Regulation by p53

In 1979, the p53 protein was recognized originally as an oncogene [20]. Later in 1989, its tumor repressor character was discovered [18]. As a tumor suppressor protein, p53 (53 KD protein), blocks cell cycles, causes death in damaged cells, and prevents proliferation in cancer cells. Inability of p53 to induce cell death results in proliferation of cells and tumor development [21].

In normal cells, the p53 protein level is maintained at a low level via proteasomal degradation by the E3 ubiquitin ligase Mdm2 [22]. Stress signals such as oxidative stress and DNA damage increase p53 protein level by deactivating Mdm2, thus leading to apoptosis. When p53 level increases, cell growth is inhibited to block the replication of damaged DNA and proliferation of cells with damaged DNA [23].

Depending on the p53's location in the cell (nuclear or cytoplasm) or based on the stress condition and type of cell, p53 can either induce or inhibit autophagy. Therefore, regulation of autophagy by p53 can result in the survival or death of the cell under chronic conditions [24].

Autophagy activation by nuclear p53 is through mTOR pathway inhibition [25]. mTOR is a growth marker and inhibitor of autophagy [25]. Therefore, autophagy activation by p53 is to protect cells from proliferation until the condition of cells are steady.

Autophagy inhibition by cytoplasmic p53 is through localization of p53 on mitochondria membrane and causing mitochondrial $\Delta\psi m$ loss and mPTP alterations [26].

Also, p53 prevents autophagy throughout prolonged starvation by decreasing level of autophagy regulators, such as LC3 and Beclin1 [27]. Phosphorylation of p53 at Ser15 causes the p53's detachment from MDM2, resulting in autophagy inhibition and apoptosis activation [28].

Depending on p53's activation signal, p53 can control the autophagy switch to apoptosis by regulating the expression of autophagy genes [29] and apoptosis genes, such as Bcl2, PUMA, Bax, etc. [29].

2.3.2. Autophagy Regulation by Bnip3

Bnip3 (Bcl2 family 19-KDa interacting protein) promoter is always repressed under basal conditions. However, in the case of hypoxia (HPX) and ischemic injury, the Bnip3 promoter is activated and can cause autophagy and apoptosis in cardiomyocytes [30]. Binp3 dual function depends on phosphorylation of C-terminus which inhibits apoptosis without blocking autophagy [31].

When Bnip3 is expressed in the cell, it attaches to mitochondrial membrane and causes the $\Delta\psi m$ loss, ROS level increase, and then swallowing of the mitochondria. This whole procedure induces mitochondrial fission and autophagy [31]. Autophagy caused by Bnip3 clears damaged mitochondria by activating mitochondrial fission through the translocation of Drp1 and Parkin to mitochondria [17]. Also, autophagy induced by Bnip3 can be prevented by inhibition of Drp1 and expression of Mfn1 [15].

2.4. Model of regulation of Autophagy by p53 in Cardiomyocytes

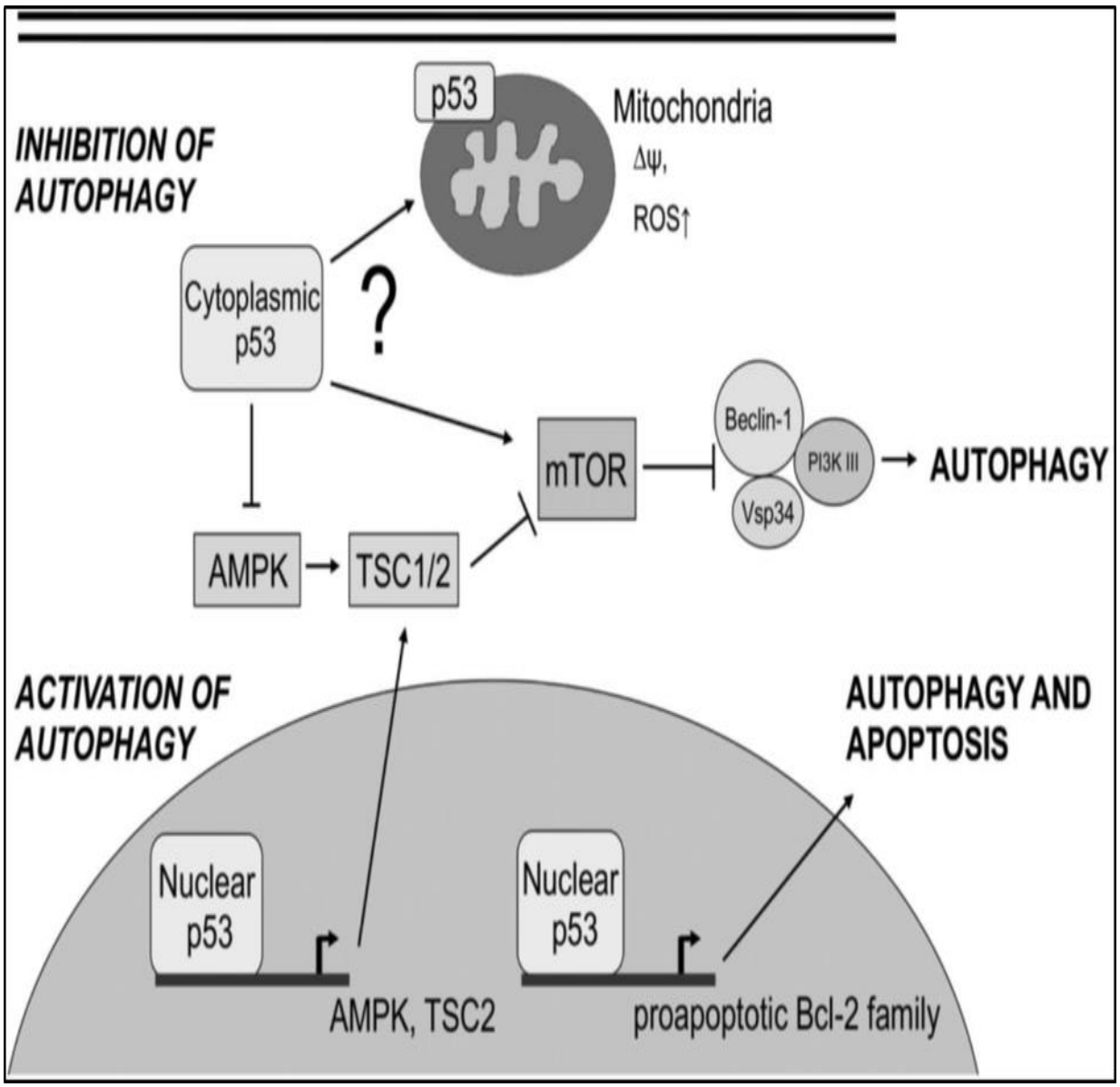


Figure 1. Autophagy Pathway regulated by p53 in cardiac cells [30]. Figure shows that p53 in cardiomyocytes has a dual role in autophagy depending on its location, whether is in cytoplasm or nucleus. When p53 is located in the cytoplasm, it inhibits autophagy by activating mTOR or inhibiting AMPK. In contrast to cytoplasmic p53, nuclear p53 activates autophagy through AMPK, TSC2, and inhibition of mTOR genes.

$\Delta\psi m$ is the mitochondrial membrane potential, ROS is the Reactive Oxygen Species, TSC2 is the Tuberous Sclerosis Protein 2, mTOR is the mammalian Target Of Rapamycin, PI3K III is the Type-III PI3 kinase.

3. Methods and Materials

3.1. Primary Cell Culture and Infection

Neonatal cardiomyocytes were isolated from the heart of one to two-day old Sprague-Dawley rat [33]. Primary cell culturing was done in serum free media as described in [33]. Myocytes were plated on 35mm plate (1×10^6 cells) and incubated with Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (DF) with addition of 17mM HEPES, 3mM NaHCO3, 2mM L-glutamine, 50µg/mL gentamicin, and 10% fetal bovine serum (FBS) in CO_2 incubator overnight at 37℃ as reported previously [33]. On the next day, after washing cells with PBS, the media was changed to DF serum-free (DFSF) medium. After changing the media, cells were kept in DFSF for control condition. For the CMV condition, cells were infected with Ad-CMV. For DOX condition, cells were treated with 5µM DOX for 18hrs. Finally, for p53 condition, p53 virus wild type form (p53-WT) was used to infect cells, and 48hrs after infection, cell cultures were collected.

3.2. Western Blot Analysis for Protein Detection

RIPA lysis buffer (made of 1.0% deoxycholate, 140 mM NaCl, 10 mM Tris-HCl, 1% Triton X-100 and 0.1% SDS) with addition of protease inhibitors (in 1ml of RIPA: 10µl of NaF, 10µl of PI, 10µl of Na_3VO_4, and 3.4µl PMSF) was used to lyse cardiomyocytes cell culture after 48hr of infection. Cell culture plates were placed on ice with shaking for 20 minutes. Cells were then scraped from the plates with a plastic cell scraper. Cell suspensions were transferred into Eppendorf tubes and spin at 12,000 RPM for 10 minutes in a 4℃ precooled centrifuge. Protein assay was done to determine the required volume for 20-50 µg of protein cell lysates. Denatured

protein samples (boiled at 100℃ for 5 minutes) were fractionated on a 10% SDS/PAGE gel and ran firstly in low voltage (80 volts) and after 20 minutes increased to 100 volts for 1 hour. The protein was transferred from the gel to a Bio-Rad supported nitrocellulose membrane (0.45 µM pore size) at 4^{o}C for one hour at 100 volts. On western blots, to determine protein bands, membranes were stained with Ponceau red dye. Skim milk 5% in 0.1% TBST was used to block filters for 1hr on a plate shaker at room temperature. Membranes were probed overnight at 4℃ with primary antibodies bound for p53 (Cell Signaling, Cat# 9282), p62/SQSTM1 (Cell Signaling, Cat#5114), or Bnip3 (developed in-house) [34]. α–Actin (Sigma) antibody was used to check the even loading of the proteins on the gel [34]. All antibodies were used at a specified dilution (as reported on their company data sheet) in 5% BSA or 2.5% milk. Filters were washed with TBST 0.1% 3 times, 5 minutes each. Membranes were then probed for 1hr at room temperature with secondary IgG antibodies anti-rabbit or anti-mouse horseradish peroxidase (HRP) conjugated. To detect bounded proteins, a chemiluminescence (ECL) reagent (Thermo Fisher Scientific) was used.

3.3. Recombinant Adenoviruses

Neonatal ventricular myocytes were infected in serum free media (DFSF) with human wild-type p53 (p53WT) adenovirus (Ad) for 48hrs. As a control, Ad-CMV that contains empty CMV enhancer-promoter was used. Human 293 embryonic kidney (HEK293) cells were used to harvest, titer, and purify adenoviruses as previously described [35].

3.4. Cell Viability Assay

Epifluorescent microscope and fluorescent dyes were used to measure cell viability (live-dead assay). Neonatal myocytes were stained with 2µM of calcein acetoxymethylester (calcein-AM,

Invitrogen by Thermo Fisher Scientific) and 2μM of ethidium homodimer-1 fluorescent dyes (VWR). Under the microscope, live cells were seen as green dots due to calcein-AM dye and the dead cells as red dots by ethidium homodimer-1 dye [36]. Neonatal cardiomyocytes were cultured on glass coverslips, treated with 5μM DOX for 18hrs, infected with p53 virus for 48hr, and incubated at dark with a mixture of both dyes for 30 minutes inside CO_2 incubator (37℃). The glass coverslips were placed on the top of glass sides. Fluorescent images of live and dead cells for each condition were then visualized under research grade epifluorescence microscope Olympus AX-70 with magnification of 200x. The Image-Pro Plus software was used to capture images. Image J program was used to count live and dead cells. Cell viability was determined by counting at least 200 cells for each tested condition with 3 replicates from 3 independent myocytes isolation. Data were then reported as the average percentage of dead cells for each condition and compared to control [37].

3.5. Mitochondrial Membrane Potential (Δψm)

Δψm in neonatal ventricular cardiomyocytes under control and overexpression of p53WT was evaluated by epifluorescence microscopy. Myocytes were grown on coverslips in DFSF media, for DOX condition, cells were treated with 5μM DOX for 18hrs and for p53 condition, cells were infected with p53 virus for 48hr. Then, cells were incubated with 50 nmol/L of florescent reagent tetra-methylrhodamine methyl ester perchlorate (TMRM) [38] (Molecular Probes, Eugene OR) for 30 minutes at 37℃ in CO_2 incubator in dark. The glass coverslips were placed on top of the glass slides and fluorescent images of each condition were captured with research grade epifluorescence microscope, Olympus AX-70, with 600x magnification. From 3 independent cardiomyocyte isolations, at least 200 cells were tested.

3.6. mPTP Opening Assay

To visualize mitochondrial permeability transition pore (mPTP) opening, Olympus AX-70 research grade epifluorescence microscope with 600x magnification and fluorescent dye were used. Neonatal cardiomyocytes were cultured on coverslip. for DOX condition, cells were treated with 5μM DOX for 18hrs and for p53 condition, cells were infected with p53 virus (p53-WT) for 48hr. The media was then extracted, and a fresh DFSF media with 5μM calcein-AM [39] (Molecular Probes) was added. 2–5μM cobalt chloride were then added to coverslips and incubated for 30 minutes in CO_2 incubator at 37℃ in dark. The formation of mPTP was observed as a reduction in the intensity of mitochondrial calcein staining. Image J software was used to measure the individual cell's fluorescence intensity of calcein-AM (green).

3.7. ROS Assay

To observe reactive oxygen species (ROS) production, neonatal cardiomyocytes were infected with adenovirus encoding CMV or p53 for 48hr and for DOX condition, cells were treated with 5μM DOX for 18hrs. Cells' media was then changed to a fresh DFSF media by adding 500 μl of 2.5 μM dihydroethidium (Molecular Probes) and then kept for 30 minutes at 37℃ in the incubator. Cells were visualized by epifluorescence microscopy. The ROS production was observed as increased intensity of red color dye.

3.8. Mitochondrial Oxygen Consumption Rate

The Agilent Seahorse XFe96 analyzer can measure the mitochondrial oxygen consumption rate (OCR) in real time. Neonatal cardiomyocytes were cultured in 96-well seahorse plates in DFSF media. A day before the assay, sensor cartridge in Seahorse XFe Calibrant was hydrated by

pipetting 190µl of calibrant media in each well and incubated at 37℃ in a non-CO2 incubator overnight. On the day of assay, seahorse assay medium was prepared by adding seahorse XF Base Medium, Na pyruvate (Gibco) 1mM, and D-glucose 10mM solutions, and pH adjusted to 7.4 with diluted NaOH. Cells were washed and incubated with the prepared assay medium for 1hr prior to the assay. 30 minutes before the assay, the calibration plate was taken out of incubator and sensor cartridges were loaded with 20µl of oligomycin (1µM), 22µl of FCCP [carbonyl cyanide4-(trifluoromethoxy) phenylhydrazone] (2µM), and 25µl of rotenone (1µM) + antimycin (1µM) inhibitor reagents in ports A, B, and C, respectively [40]. Calibration plate was then incubated for 2-3 minutes, placed on the instrument tray, and ran for approximately 15-30 minutes. After calibration plate running was completed, the cell culture microplate was placed in the machine. Respiration data was analyzed using the Seahorse XF Cell Mito Stress Test Report Generator software. Oxygen consumption rate (OCR), Basal respiration, maximal respiration, and spare respiratory capacity were determined using this software.

3.9. Data Analysis

The comparison of mean differences between the control, CMV, DOX and p53 groups were done using One-way ANOVA. In order to show the differences among the groups, Bonferroni post hoc tests were used. At p-value less than 0.05, differences were counted to be statistically significant. In all the experiments, data were collected from 3 independent myocytes isolations, using 3 replicates under each tested condition.

4. Results

4.1. Doxorubicin treatment upregulates p53 in ventricular cardiac cells

Doxorubicin (DOX), is an antitumor drug, which is used to treat different types of cancer worldwide [37]. DOX is known for its acute and chronic cardiotoxic effects, including mitochondrial dysfunction [37]. Previous work in our laboratory showed that the p53 tumor suppressor protein caused autophagy cell death of cardiac cells [30]. Therefore, we were interested to test if DOX induces cell death through p53. To determine the involvement of p53 in doxorubicin mediated cell death, ventricular cardiomyocytes were treated with 5μM DOX for 18hrs and then cell lysate was collected for western blot analysis. As shown in Figure 2, compared to control, DOX treatment caused an increase in the level of p53 protein.

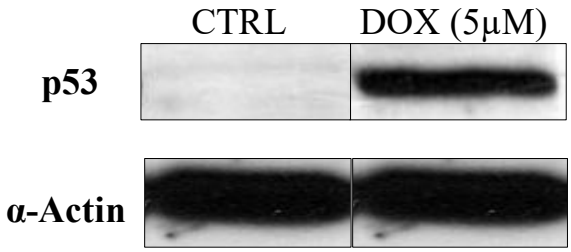


Figure 2. Doxorubicin treatment upregulates p53 expression in ventricular cardiomyocytes. Western blot comparison of p53 expression in control medium and with DOX treatment. The membrane was probed with an antibody directed against p53 as explained in method section. α–Actin antibody was used to verify the equivalent protein loading.

4.2. Doxorubicin treatment provokes mitochondrial perturbation

Mitochondria has a vital role in regulating cellular respiration and energy metabolism. Less is known about the signaling pathways that mediate the cardiotoxic effects of DOX. There are theories that DOX causes mitochondrial perturbation with mitochondrial permeability transition pore (mPTP) opening, loss of mitochondrial membrane potential ($\Delta\psi m$), and increased reactive oxygen species (ROS) production [37]. Therefore, to determine the effect of doxorubicin on mitochondria, cultured neonatal cardiomyocytes were treated with 5μM DOX 18hr prior to fluorescent microscopy. ROS, PT-pore (permeability transition pore), and $\Delta\psi m$ assays were done as can be seen in Figure 3. DOX treatment compared to control caused more ROS production, more pore opening, and lose of membrane potential.

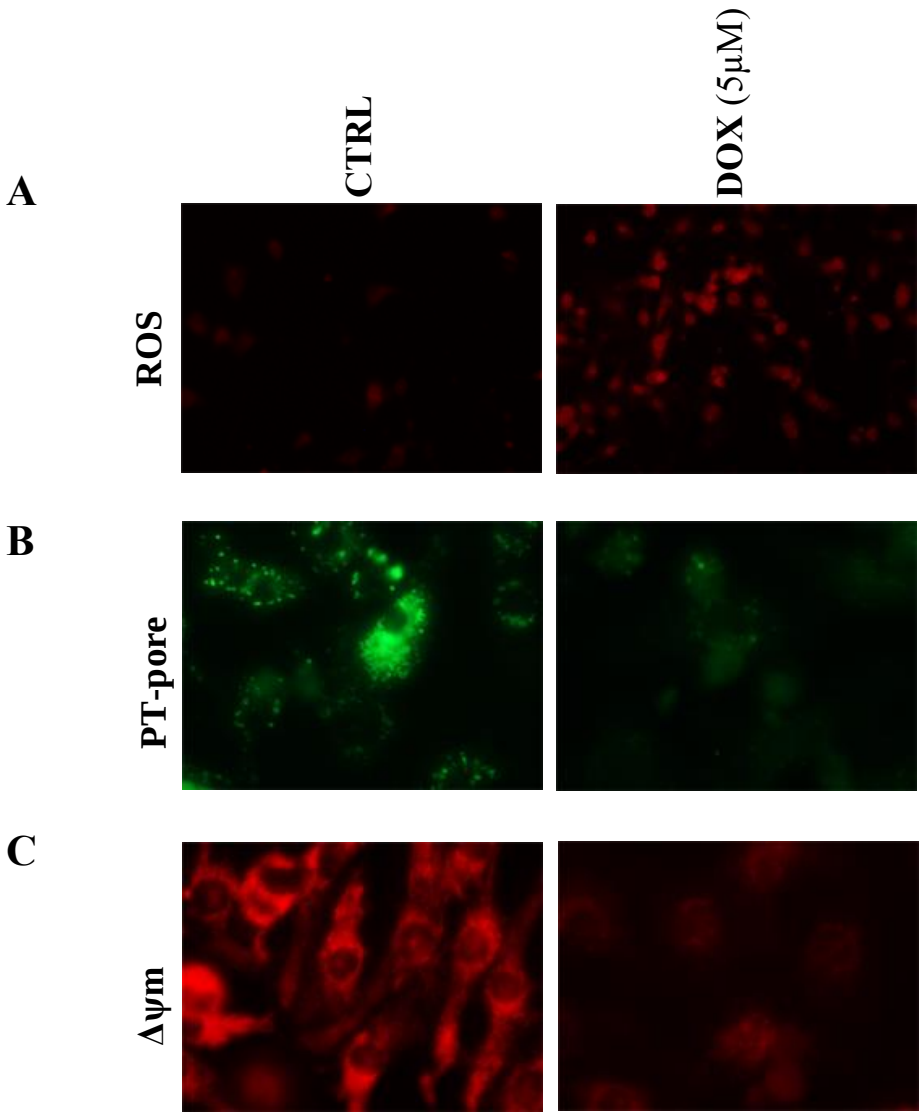
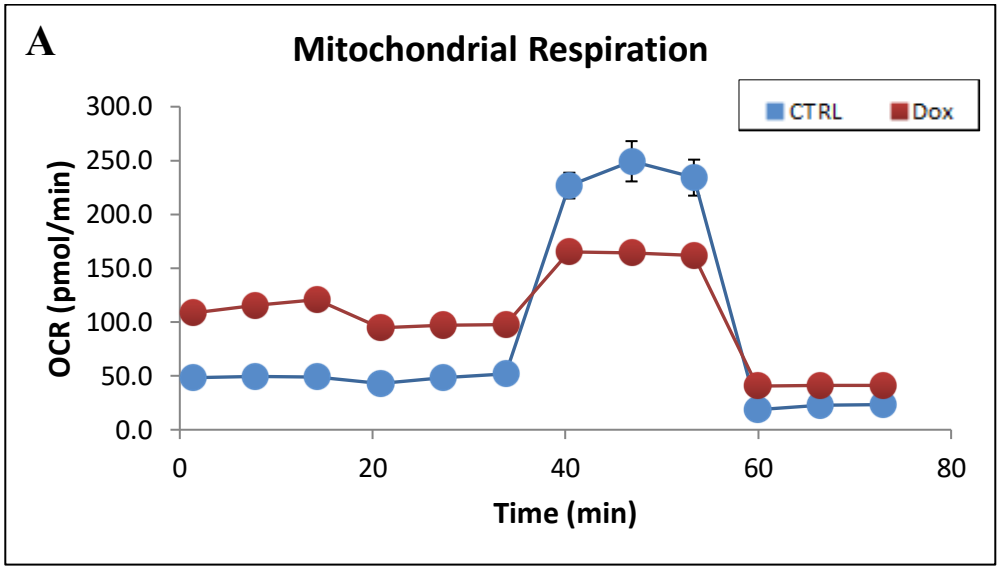
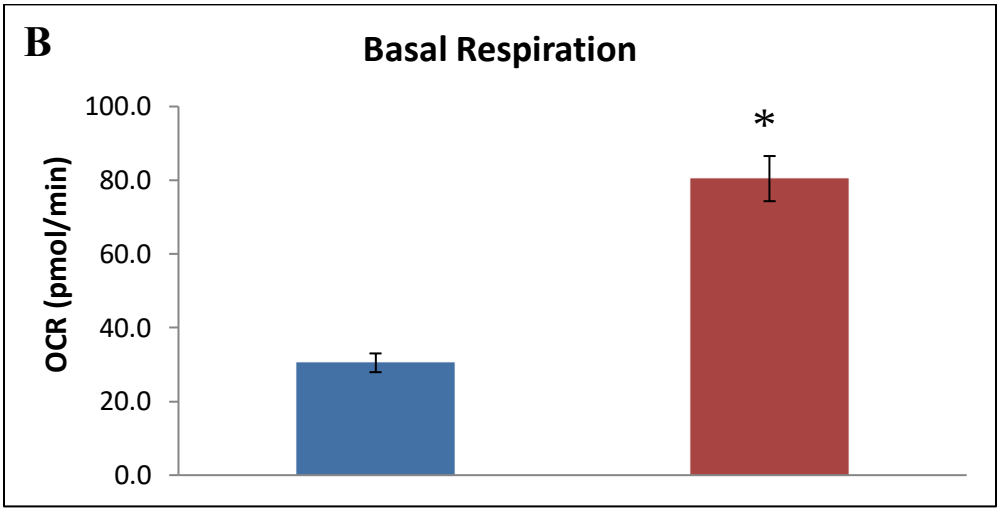


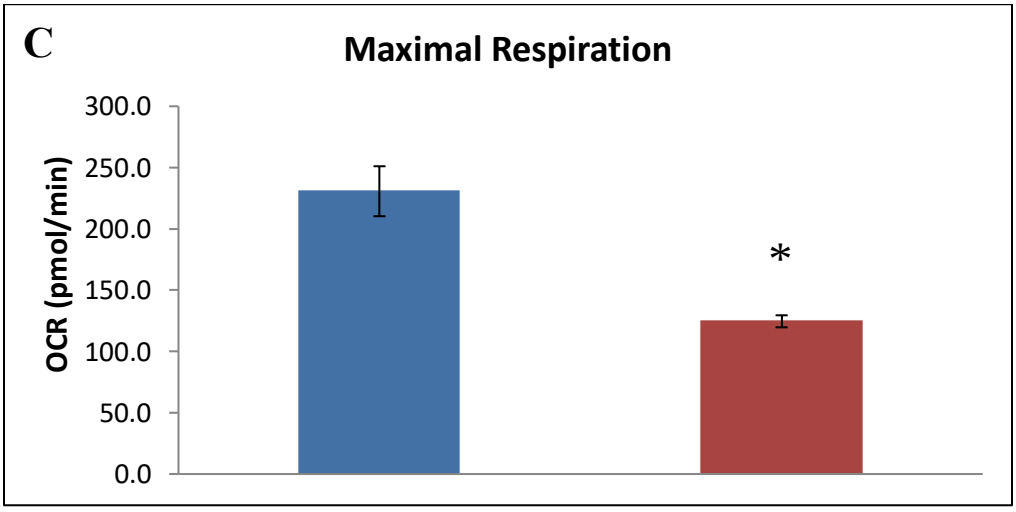
Figure 3. Doxorubicin treatment causes mitochondrial perturbation of cardiac cells. Epifluorescence microscopy images of CTRL and DOX treated cardiomyocytes for ROS assay (panel A), PT-pore assay for mitochondrial permeability transition pore opening (Panel B) and mitochondrial Δψm (panel C) as explained in Materials and Methods section of thesis.

4.3. Doxorubicin treatment has effect on cardiac cells respiration

Mitochondria are very important for oxidative metabolism. Therefore, to maintain mitochondrial membrane potential electron transport chain complexes, which are located on the inner mitochondrial membrane (IMM), are very vital. Transfer of electrons through these complexes is very important for forming the proton gradient and electromotive force for maintaining $\Delta\psi m$. Mitochondrial perturbation induced by DOX was observed in Figure 3 in the form of increased ROS production, mPTP opening, and loss of $\Delta\psi m$. Therefore, we can predict that DOX can cause defect to respiratory chain activity as well. To assess the possibility, the mitochondrial respiration test was done by using Agilent Seahorse XFe96 analyzer as explained in the Methods section. DOX (5μM) treatment on neonatal cardiomyocytes was done 18hr prior to the seahorse experiment. Oxygen consumption rate (OCR), maximal respiration, and spare respiratory capacity were decreased dramatically under the DOX condition compared to control as can be seen in Figure 4A, C, and D, respectively. In Figure 4B we can see a higher basal respiration in DOX treated condition compare to control because in stress condition cells try to overcome situation by more glycolysis.


A
Mitochondrial Respiration
CTRL
Dox
OCR (pmol/min)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
0
20
40
60
80
Time (min)


B
Basal Respiration
OCR (pmol/min)
100.0
80.0
60.0
40.0
20.0
0.0
*


C
Maximal Respiration
OCR (pmol/min)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
*

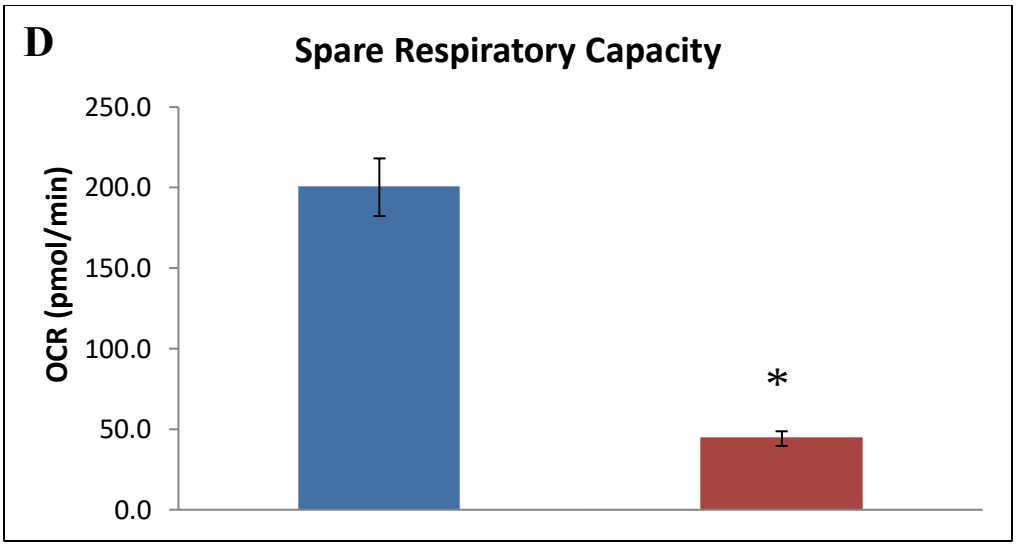


Figure 4. Doxorubicin effect on cellular respiration of cardiomyocytes. Measurement of mitochondrial function in cultured neonatal cardiomyocytes using the seahorse XFe96 analyzer. Oxygen consumption rate (OCR) was measured at basal level with the subsequent and sequential addition of oligomycin (1 µM), FCCP (2 µM) and rotenone (1 µM) + antimycin A (1 µM). Figure presents mitochondrial respiration data for CTRL (blue) and DOX (red) conditions. Panel A: graphical demonstration of OCR in cardiac cells that were treated with DOX. Histograms present quantitative data, and values are presented as mean ± SEM from five to 6 replicates with p value less than 0.05. Panel B: basal respiration, '*' DOX condition is statistically different from CTRL condition with $p<0.039$. Panel C: maximal respiration, '*'CTRL/DOX $p<0.021$. Panel D: spare respiratory capacity, '*'CTRL/DOX $p<0.016$.

4.4. p53 provokes mitochondria defect in cardiomyocytes

Our laboratory has previously shown that p53 localizes to the mitochondria of cardiomyocytes [30]. Hence, to determine if p53 can cause mitochondrial damage and cell death, we looked at mitochondrial function by ROS, $\Delta\psi m$, and PT-pore assays. As can be seen in Figure ***5***, p53 overexpression caused a dramatic increase in ROS production and loss of membrane potential by mitochondrial pore opening. CMV was used as a control for the p53 virus. These data support the idea that p53 provokes mitochondria defect in ventricular cardiomyocytes and promotes cell death.

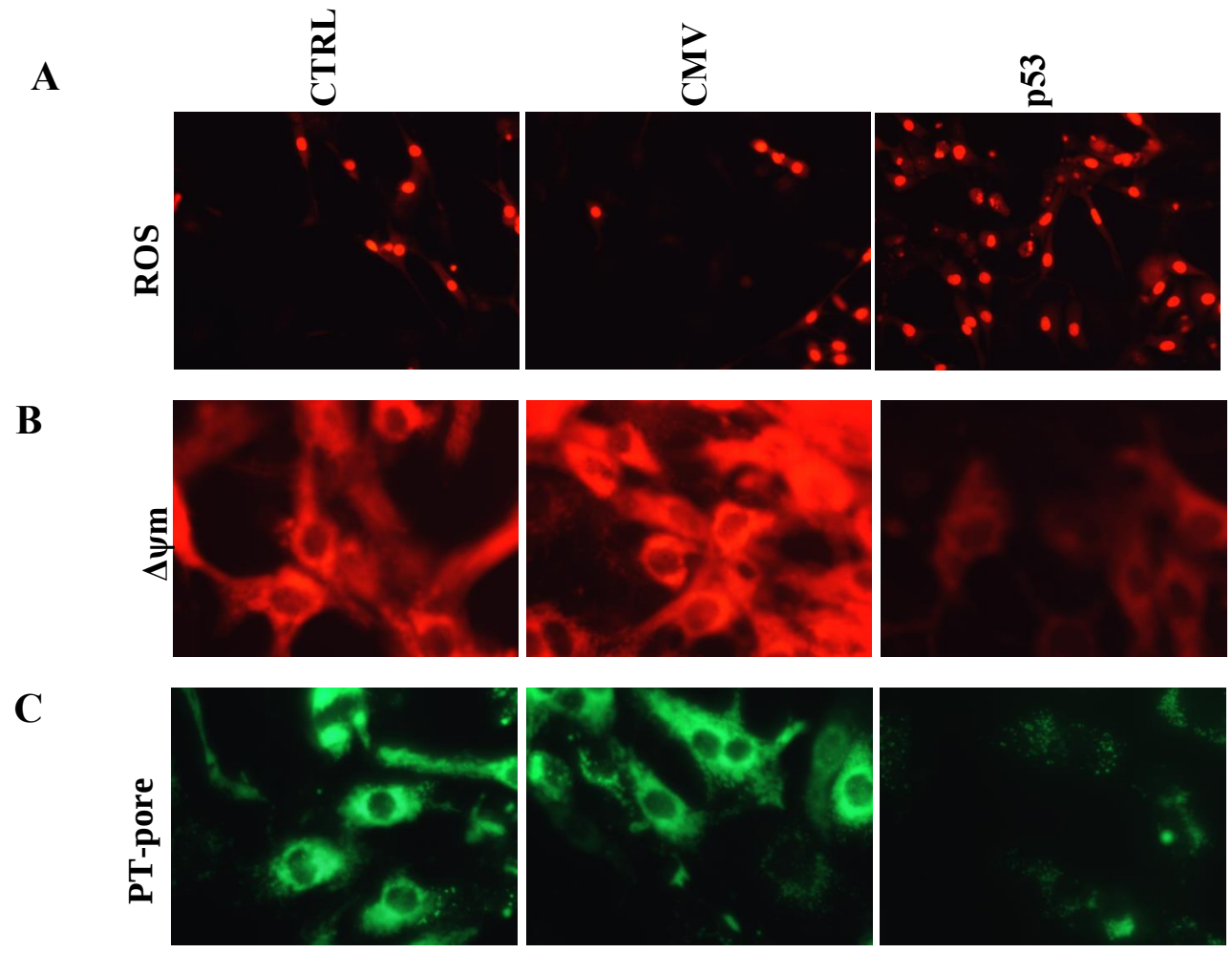

A
CTRL
CMV
p53
ROS
B
Δψm
C
PT-pore
D
(PT-pore)
% change from control
140
120
100
80
60
40
20
0
***
ns
***
CTRL
CMV
p53

Figure 5. p53 causes mitochondrial defects in ventricular cardiomyocytes. Epifluorescence microscopy images of CTRL, CMV and DOX treated cardiomyocytes for ROS assay (panel A), mitochondrial $\Delta\psi m$ (panel B) and PT-pore assay for mitochondrial permeability transition pore opening (Panel C), as explained in Materials and Methods Section. Panel D: Histogram shows a numerical data for panel C. Data are expressed as mean±S.E. from 3 independent cardiac myocyte isolations with more than 200 cells counting for each tested condition with p value less than 0.05. 'ns' donates statistically non-significant between CTRL and CMV condition. '***' indicates p value <0.0002 for p53 compared to CMV and CTRL conditions.

4.5. p53 alters autophagy process in ventricular cardiomyocytes

Autophagy under conditions of stress such as heart disease can be adaptive as a vital process to maintain homeostasis of cells by removal of accumulated macromolecular proteins and damaged organelles or be maladaptive to end cell's life. For assessing the activation of the autophagy response, we looked at autophagy markers Beclin 1 and p62. To test whether p53 can cause autophagy, neonatal cardiac myocytes were infected with adenovirus encoding CMV or p53 for 48hrs. Cell lysate was then collected for western blot analysis. In contrast with control and CMV, increase of autophagy markers Beclin1 and p62 can be seen in cardiac cells overexpressing p53 as shown in Figure 6.

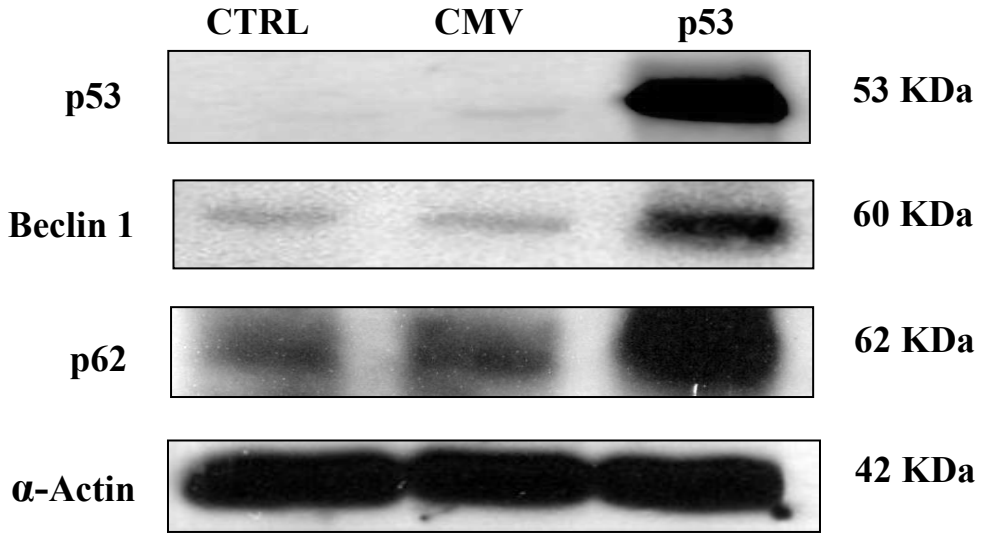


Figure 6. p53 alters autophagy markers in ventricular cardiomyocytes. Western blot analysis of cardiomyocytes lysate with overexpressing CMV or p53 adenovirus for 48hrs. The membranes were probed with antibodies directed against p53, Beclin 1, p62. α–Actin served as control for equal protein loading.

4.6. p53 expression increases cell death of ventricular cardiomyocytes

Western blot data shown in Figure 6 demonstrated that the level of autophagy marker p62 was increased in cardiac cells upon p53 overexpression. p62 acts as a receptor for autophagy that connects the autophagosome complex to its' degradation cargo. To establish the role of p53 in autophagic cell death, ventricular cardiomyocytes were infected with adenovirus encoding CMV or p53 for 48hrs (CMV was used as a control for the virus). Then, cell viability assay was done by fluorescent dyes calcein-AM and ethidium homodimer-1. Epifluorescence microscopy revealed that p53 caused excessive cell death compared to control and CMV as can be seen in Figure 7A. The live cells were observed in green due to calcein-AM dye and the dead cells in red by ethidium homodimer-1.

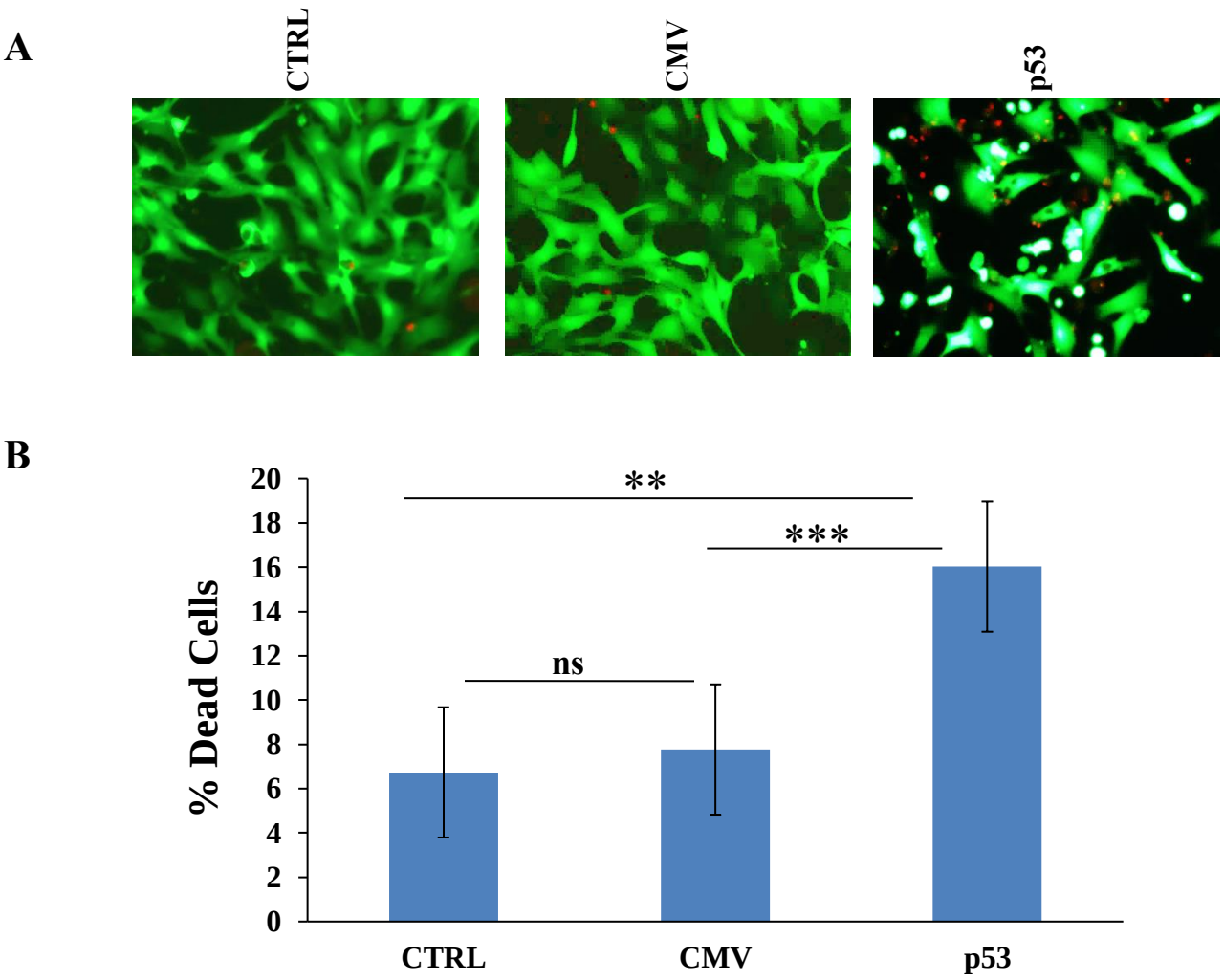
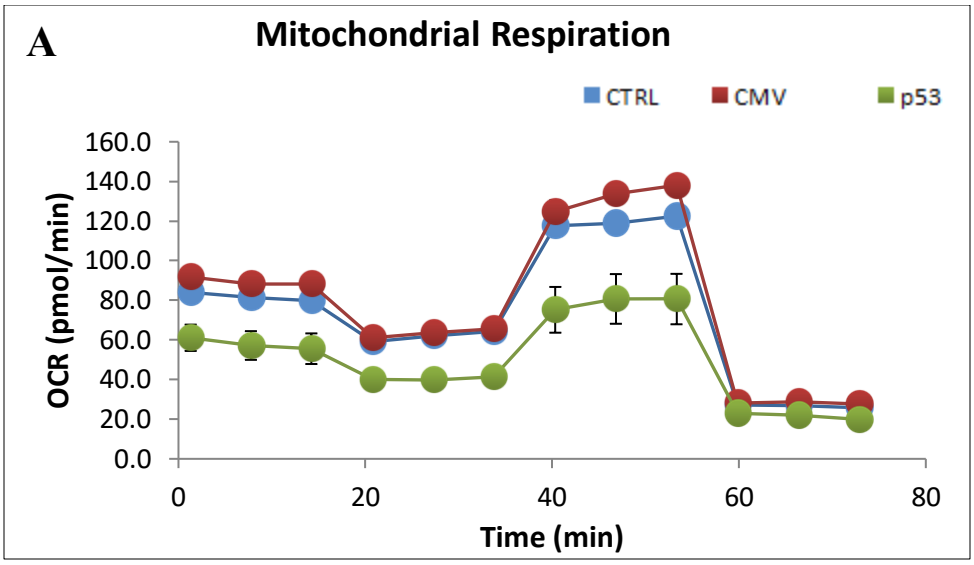
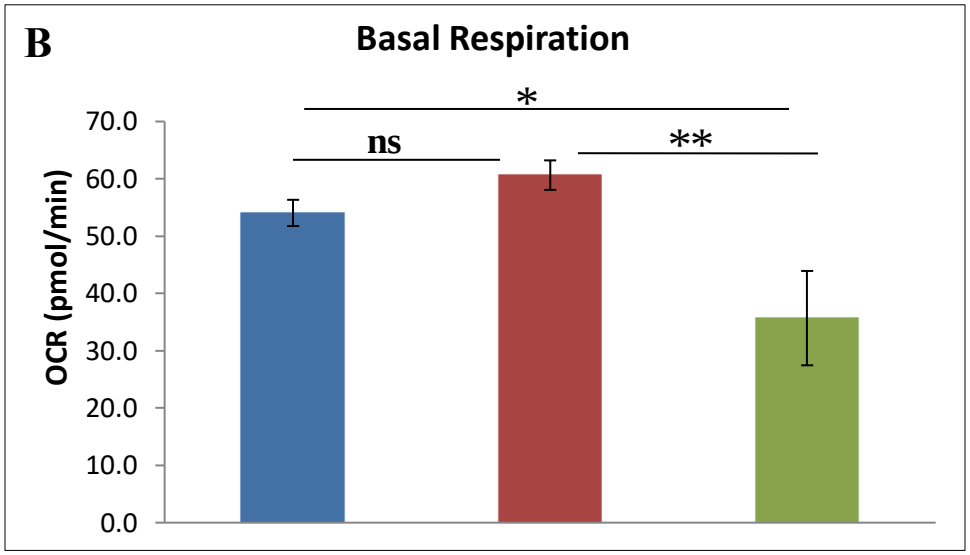


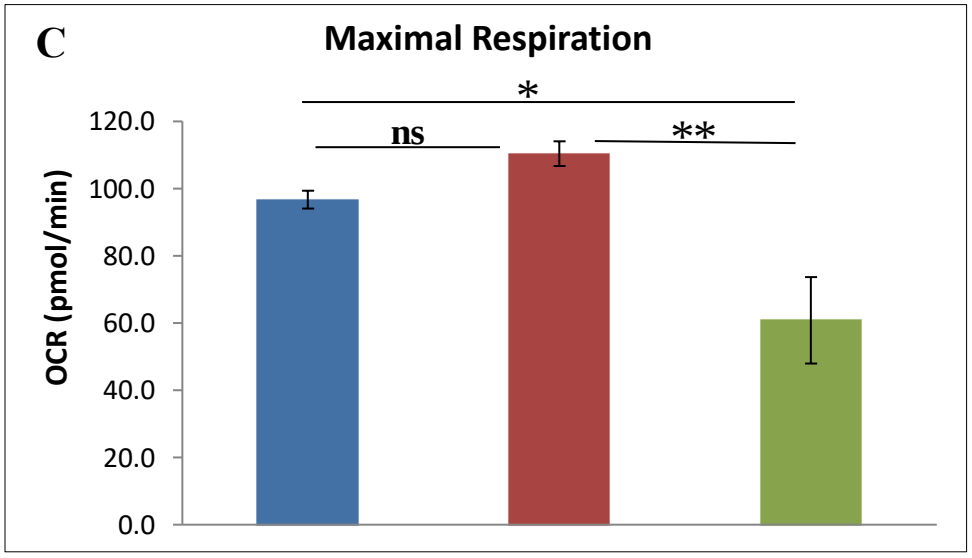
Figure 7. p53 expression increases cell death of ventricular cardiomyocytes. Panel A: primary cell culture of neonatal cardiomyocytes was infected with adenovirus encoding CMV or p53 for 48hrs and then imaged by epifluorescence microscopy. Cardiomyocytes were stained with vital dyes calcein -AM and ethidium homodimer-1 to detect the number of dead cells (red) and live cells (green). Panel B: Histogram shows a numerical data for panel A. Data are expressed as mean±S.E. from 3 independent cardiac myocyte isolations with more than 200 cells counting for each tested condition with p value less than 0.05. 'ns' donates statistically non-significant between CTRL and CMV condition. '**' denotes p53 compared to control condition with p value <0.0003. '***' indicates p value <0.0005 for p53 compared to CMV condition.

4.7. p53 overexpression disrupts mitochondria oxidative metabolism

p53 causes mitochondrial defect as shown in Figure 5. We wanted to investigate the effect of p53 on mitochondrial activity, since mitochondria is very important in oxidative metabolism. Therefore, a Seahorse experiment was performed to measure oxygen consumption rate of ventricular cardiomyocyte after 48hrs treatment with adenovirus encoding p53 or CMV. As it can be seen in Figure 8, OCR decreased under the p53 condition compared to the controls.


A
Mitochondrial Respiration
CTRL
CMV
p53
OCR (pmol/min)
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
20
40
60
80
Time (min)


B
Basal Respiration
*
ns
**
OCR (pmol/min)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0


C
Maximal Respiration
*
ns
**
OCR (pmol/min)
120.0
100.0
80.0
60.0
40.0
20.0
0.0

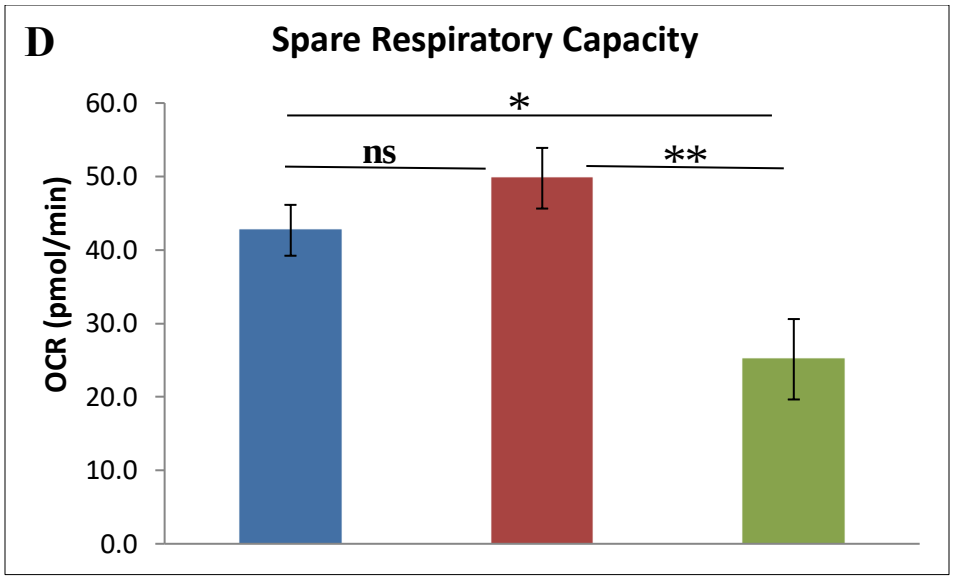


Figure 8. p53 overexpression interrupts oxidative metabolism of mitochondria. Measurement of mitochondrial function in cultured neonatal cardiomyocytes using the seahorse XFe96 analyzer. OCR was measured at basal level with the subsequent and sequential addition of oligomycin (1 µM), FCCP (2 µM) and rotenone (1 µM) + antimycin A (1 µM). Histograms present quantitative data for basal and maximal respiration and spare capacity which Data are presented in as mean ± SEM from five to six replicates with p value less than 0.05, and 'ns' donates statistically non-significant between CTRL and CMV condition. Panel A: shows measurement of respiration (OCR) in cardiac cells that were infected with adenovirus encoding CMV (red) or p53 (green). Panel B: basal respiration, '*'CTRL vs. p53 with $p<0.048$, and '**'CMV vs p53 $p<0.007$. Panel C: maximal respiration, '*'CTRL/p53 $p<0.01$, '**'CMV/p53 $p<0.001$. Panel D: spare respiratory capacity, '*'CTRL/p53 $p<0.04$, '**'CMV/p53 $p<0.004$.

4.8. p53 shifts metabolism toward glycolysis

Pyruvate dehydrogenase (PDH) is a large enzyme located on the mitochondria matrix. It converts pyruvate to acetyl CoA as an end product of glycolysis, while it is required to start the TCA cycle. PDH is a vital enzyme, which connects two main metabolic pathways: glycolysis and the TCA cycle. When PDH is phosphorylated, its activity decreases, and metabolism shifts to glycolysis.

p53 causes mitochondrial defect, and therefore, we hypothesized that p53 can cause a shift in metabolism of cardiomyocytes from oxidative metabolism to glycolysis. To test this hypothesis, cardiomyocytes were infected with adenovirus coding CMV or p53 virus for 48hrs. Cell lysate were then collected for western blot analysis. As it can be seen in Figure 9, p-PDH was increased with p53 overexpression compared to control and CMV.

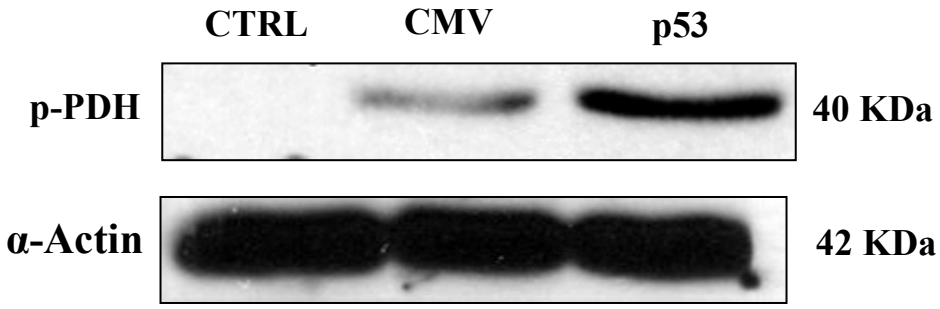


Figure 9. p53 causes metabolism shift to glycolysis. Membrane was probed with antibody against the phospho-pyruvate dehydrogenase (p-PDH). α–Actin served as control for equal protein loading.

5. Discussion

Doxorubicin, as a chemotherapeutic agent, is used worldwide to treat different types of human cancers. However, the molecular mechanisms regarding its cardiotoxic effects, such as mitochondria defects are still ambiguous. Previous work from our laboratory has shown that p53 tumor suppressor causes autophagy cell death in cardiomyocytes [30]. Therefore, we wanted to understand if DOX causes cell death through p53. The data in Figure 2 shows that DOX treatment upregulates p53 expression in ventricular cardiomyocytes. Evidence such as increased ROS production, mPTP opening, and lose of membrane potential ($\Delta\psi m$) in Figure 3 supports the model that DOX causes mitochondrial perturbation and cell death in cardiac cells.

Mitochondria have an essential role in regulating cellular respiration and energy metabolism. Since DOX can cause mitochondria perturbation, we predicted that it also can defect respiratory chain activity as well. This idea was supported by data from the seahorse experiment using the Agilent Seahorse XFe96 analyzer. Neonatal cardiac myocytes were treated with DOX (5μM) 18hr before seahorse experiment was done. Oxygen consumption rate (OCR) declined dramatically in DOX condition compared to control, as can be seen in Figure 4.

Autophagy is a catabolic pathway, which is performed to remove damaged or excessive organelles, such as mitochondria. It has an important role in growth and survival. Autophagy by lysosomal degradation of the cytoplasmic structures recycles nutrient and energy (ATP) during cell starvation. Under normal conditions, autophagy level is low, but increases immediately when situations such as starvation or stress happens [12]. Autophagy needs to be tightly regulated, otherwise it will lead to human diseases, such as heart failure, cancer, and neurodegenerative diseases [13]. Autophagy can be adaptive and protects cells and maintains hemostasis. However,

it can also be maladaptive and causes cells death. Although less autophagy can be dangerous for cell, too much autophagy can damage the mitochondria and leads to cell injury or death.

Previous studies from our laboratory have shown that p53 tumor suppressor localizes to mitochondria of cardiac cells and causes defect, which is consistent with epifluorescence microscopy data of neonatal cardiomyocytes by ROS, $\Delta\psi m$, and PT-pore tests shown in Figure 5. Also, the cell viability data shown in Figure 7 supports the hypothesis that p53 causes mitochondrial perturbation which leads to cell death. Western blot analysis Data shown in Figure 6 indicates that p53 causes autophagy cell death (maladaptive autophagy) in cardiac myocytes. By overexpression of p53 in cardiac cell for 48hr, cell lysates were collected for western blot analysis and probed with antibodies against autophagy markers, Beclin 1 and p62. protein Beclin 1, as an autophagy marker required for autophagosomes formation, supports the idea that p53 caused autophagy in cardiomyocytes. p62 protein get activated in autophagy to connect autophagosomes to degradation cargo. This supports the idea that p53 caused maladaptive autophagy in cardiac myocytes.

As data presented in Figure 5 demonstrate, p53 causes mitochondrial defect. Therefore, it can be predicted that mitochondrial activity would be affected as well, since mitochondria is very important in oxidative metabolism. Therefore, seahorse experiment was done to measure OCR of ventricular cardiac myocytes with p53 overexpression. As it can be seen in Figure 8, OCR was declined in p53 condition compared to controls.

The pyruvate dehydrogenase (PDH) is located on the matrix of mitochondria. It converts pyruvate to acetyl CoA, which is the end-product of the glycolysis and needed for TCA cycle to start. When PDH get phosphorylated, its activity decreases, and metabolism shifts to glycolysis. Therefore, data in Figure 9 supported that p53 induced a shift in metabolism of cardiomyocytes

from oxidative to glycolysis that is dependent upon p53 mediated mitochondrial defects. The western blot data, shown in Figure 9, demonstrates that p-PDH was increased with p53 overexpression in cardiomyocytes compared to controls.

Results of this thesis demonstrate that p53, as the inducer, links mitochondria perturbation to autophagic cell death of cardiomyocytes. p53 changes mitochondrial permeability causing loss of membrane potential, which is supported by our data. This leads to the activation of mitophagy and the clearance of damaged mitochondria from the cell.

Knowledge about autophagy and p53 signaling pathway in cardiac myocytes would be beneficial to find therapeutic ways to control p53 expression in order to reduce cell death in cardiac diseases.

6. Future Directions

There are several remaining unanswered questions regarding the signaling pathways that connect autophagy to different types of cell death, and its dual role for rescuing cells or causing cell death. Most research studies regarding autophagy and p53 are performed in human cancer cells and mutated cell lines, but not in cardiomyocytes. These types of cell lines have deficiencies in the cell death pathways and mutations in tumor suppressor genes such as p53. First, it is important to study differences in autophagy response in different cell lines under different stress conditions. Secondly, more studies are required to demonstrate how autophagy switches from being adaptive to maladaptive in cardiomyocytes. Finally, further studies are also necessary to indicate the regulation of autophagy in cardiac cells by p53.

Alongside the future studies, knowledge on how autophagy regulates cardiac cells at cellular and molecular levels would be beneficial for decreasing the impact on cardiac diseases, such as heart failure induced by doxorubicin.

7. Conclusion

Data in this thesis demonstrate that p53 causes mitochondrial perturbations as shown by increased ROS, PT-pore, $\Delta\psi m$, and viability assays. We demonstrated that p53 causes autophagy of ventricular cardiomyocytes which shifts autophagy from cell survival to cell death. As it can be seen by data from the western blot analysis, overexpression of p53 increases the level of Beclin1 and p62. This thesis demonstrates that autophagy and cell death are connected in cardiac myocytes treated with doxorubicin.

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