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Inhibition of mTOR Signaling Protects Against Myocardial Reperfusion Injury, Acute Myocardial Infarction

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INHIBITION OF mTOR SIGNALING PROTECTS AGAINST MYOCARDIAL REPERFUSION INJURY

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at Virginia Commonwealth University.

by

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Abstract

INHIBITION OF mTOR SIGNALING PROTECTS AGAINST MYOCARDIAL REPERFUSION INJURY

By Scott Michael Filippone, M.S.

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Acute myocardial infarction (AMI) is the leading cause of death worldwide. Currently, the best method of treating cardiac ischemia is early reperfusion which, itself, induces myocardial damage. The mTOR complex is a key regulator of cardioprotection against cell stressors. We hypothesized that reperfusion therapy with Rapamycin, a potent mTOR inhibitor, would reduce infarct size in adult mouse hearts. Rapamycin was administered at the onset of reperfusion following 30 min *in situ* LAD ligation. After 24

hours of reperfusion, myocardial infarct size and apoptosis were significantly reduced in rapamycin-treated mice compared to control. Rapamycin inhibited pro-apoptotic protein Bax and phosphorylation of ribosomal protein S6 (target of mTORC1), while it induced phosphorylation of AKT (target of mTORC2). Rapamycin also induced phosphorylation of ERK, while significantly reduced phosphorylation of p38. Thus, our study shows that reperfusion therapy with Rapamycin provides cardioprotection through induction of the phosphorylation of Akt and ERK.

Introduction

1.1 Ischemic Heart Disease

Of the vast amount of tissue types in the human body, few respond as adversely to hypoxic conditions as cardiac muscle. Originally thought to only be a major issue in developing nations, ischemic heart disease was identified as the world's leading cause of death in 2010.[1] In addition to the staggering loss of life to initial myocardial infarction (MI), individuals who survive a heart attack see a dramatic decline in cardiac function, often resulting in heart failure (Figure 1). Patients such as these become dependent on medical interventions for the rest of their lives, taking a tremendous economic toll on the healthcare system; as the world's population has increased, so too has this burden.[2] Thus, it is of no surprise that cardioprotective therapies have garnered a great deal of attention in the scientific community. To date, prompt reoxygenation of ischemic tissue has proven to be the most effective method of minimizing infarct size; a process known as reperfusion.[3,4] However, this treatment brings with it it's own set of challenges in the form of infarct-forming reperfusion injury.[5] The key, then, is to identify the process by which this injury occurs, and how to best reduce its negative impact on the myocardium.

1.2 Mechanisms of Ischemic Injury

Ischemia/Reperfusion injury (I/R) is best characterized by the infarct region produced by both the ischemic state of the myocardium, and the damaging effects of reperfusion therapy. Ischemia occurs when a tissue is not perfused properly, resulting in

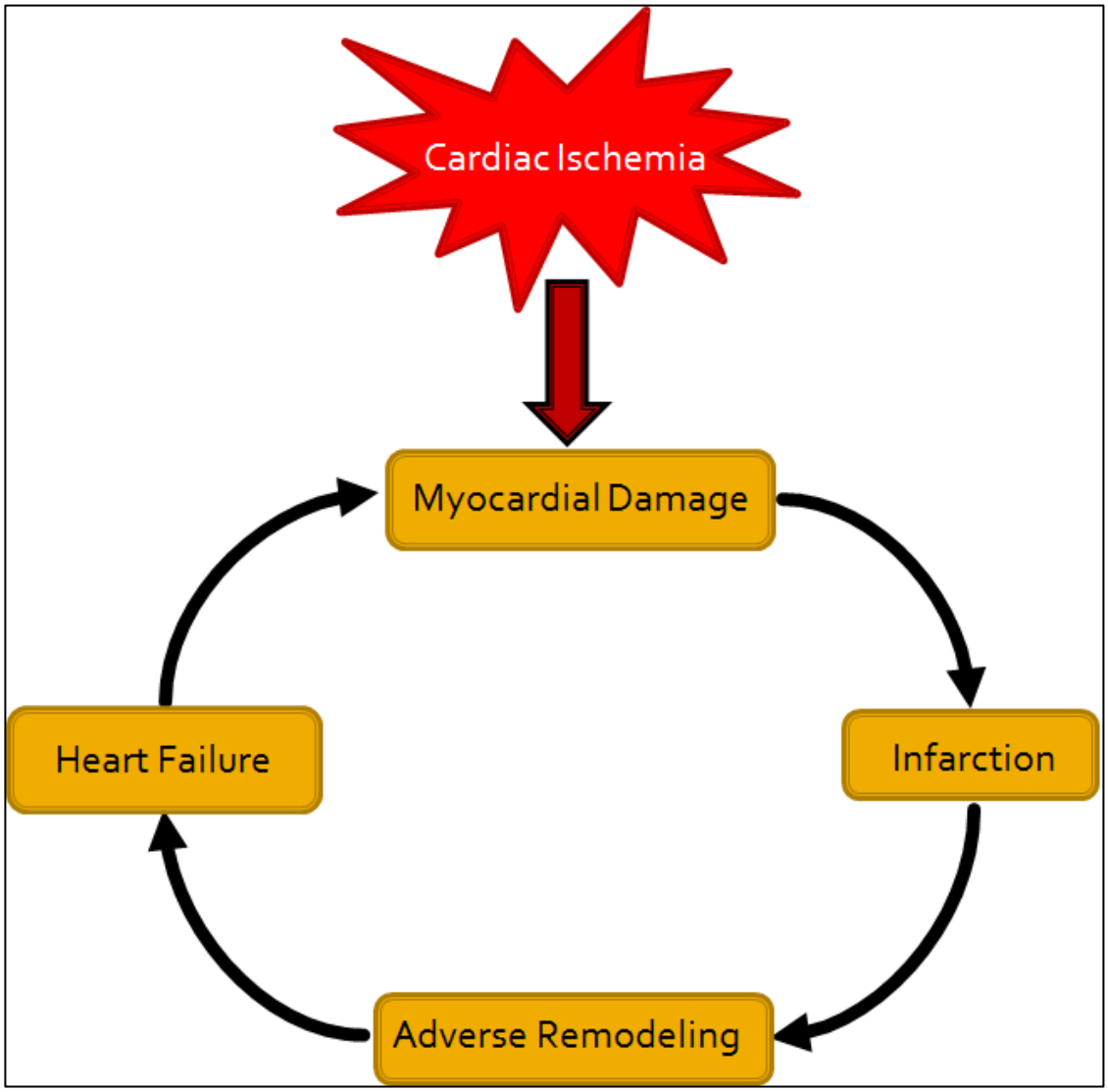

Figure 1. Complications following cardiac ischemia. The lethal downwards spiral that can arise from a single ischemic event in the heart. The myocardial damage produced by ischemia creates a functionless infarct region within the myocardium. As initial attempts to compensate for the damage fail, the heart may undergo adverse remodeling processes, resulting in cardiomyopathies that impede cardiac function. When the heart is no longer able to properly function, the patient suffers from heart failure, which can promote further opportunities for cardiac damage.

inadequate oxygenation of said tissue. In the heart, this is most commonly due to coronary artery occlusion arising from hardened plaque deposits that accumulate on the arterial wall over time (atherosclerosis).[6] Because these vessels are responsible for distributing oxygenated blood throughout the myocardium, their occlusion results in the body's primary blood pumping organ being starved of oxygen.

Cardiomyocytes are unique in their extraordinary level of contractile activity. Barring pathologic conditions, these cells work ceaselessly and, as such, have massive energy demands. In order to supply themselves with the vast amount of ATP required for their constant function, their cytosol is highly mitochondria-dense.[7,8] Ordinarily, oxygen functions as the terminal electron acceptor of the electron transport chain (ETC) located within the inner mitochondrial membrane. The reduction of oxygen to water is integral in establishing the proton gradients required for oxidative phosphorylation and proper ATP production.[7,8] When the cell becomes hypoxic, aerobic respiration is no longer available, and the mitochondria falls back to less efficient anaerobic glycolysis, initiating a cascade of events that defines ischemic injury. Lactate, a by-product of this cycle, is an acidic compound that begins to flood the cytosol, decreasing the intracellular pH of the myocyte. The ensuing increase in $[H^+]_i$ serves to drive the cell membrane-bound Na^+-H^+ antiporter to draw an influx of Na^+ ions into the cell. Under physiologic conditions, these Na^+ ions would simultaneously be transported back out by the $3Na^+$-$2K^+$-ATPase, but inadequate ATP generation dictates otherwise. Thus, an alternate compensatory mechanism is activated by an additional membrane-bound antiport channel: The $2Na^+$-Ca^{+2} exchanger.[5,7-

[10] The net result is a massive $[Ca^{+2}]_i$ spike under ischemic conditions. While anaerobic glycolysis serves as a 'fallback' pathway during hypoxia, by no means it adequately supplies the cardiomyocyte with enough ATP to properly function. Consequently, basic metabolic needs are not met, and degradative enzymes such as proteases and phospholipases further inhibit mitochondrial function. A rapid decline in cellular integrity ensues, as ionic gradients are no longer able to be maintained, and contracture ceases. Left unchecked, ischemia will continue indefinitely, causing irreparable damage to the heart that will prevent proper function.

1.3 Mechanisms of Reperfusion Injury

Early reoxygenation of the heart, known as reperfusion, is currently the most effective therapeutic method of treating patients suffering from ischemia.[11,12] While it is a necessary and definitive end to the ongoing ischemia, reperfusion imposes it's own set of infarction-producing challenges. In this sense, reperfusion therapy functions as a doubled-edged sword: It is necessary to avoid the near-certain death afforded by ischemia, yet it must be controlled carefully to minimize further infarct size progression.[12] In order to fully understand the mechanisms of reperfusion injury, it is first important to realize the importance of the Mitochondrial Permeability Transition Pore (MPTP) and how it ties into both programmed and un-programmed cell death (apoptosis and necrosis, respectively).

The MPTP, normally closed, serves to establish selective membrane permeability of the inner mitochondrial membrane, while simultaneously establishing the ionic

gradients necessary for proper ETC function. Conditions of oxidative stress, ATP depletion, and Ca^{+2} overload are conducive to MPTP opening; these pathologic states define ischemia, yet MPTPs generally remain closed during such an episode. Coincidentally, the acidic cytosol, resulting from increased lactic acid, inhibits MPTP opening. It isn't until reoxygenation of the tissue that said lactic acid is 'washed out', and conditions become ideal for the opening of MPTPs. As these pores open, calcium floods the inner mitochondrial matrix and the resulting increase of osmotic pressure causes the mitochondria to swell. Matrix swelling strains and, eventually, ruptures the outer mitochondrial membrane, initiating a release of cytochrome c and pro-apoptotic proteins, such as Bax, that lead to caspase activation and eventual cell death (Figure 2).[7-10,12-15] Of particular interest are the pro-apoptotic Bax protein and the anti-apoptotic Bcl protein family. Bax exists within the cytosol and, when exposed to apoptotic conditions, oligomerizes and inserts itself into the outer mitochondrial membrane; thus, facilitating a release of additional apoptotic factors.[3] Recent research has also elucidated the role of the Bcl-2 member of the Bcl family of anti-apoptotic proteins. Not only has Bcl-2 depletion been proven to cause apoptosis by a similar mechanism as Bax, but Bcl-2 inhibition has been shown to sensitize MPTPs to opening, indicating that it's induction is an important part of defending against cell death.[4] Indeed, both of these proteins have demonstrated their potential for functioning as future therapeutic targets to minimize infarct size due to programmed cell death.

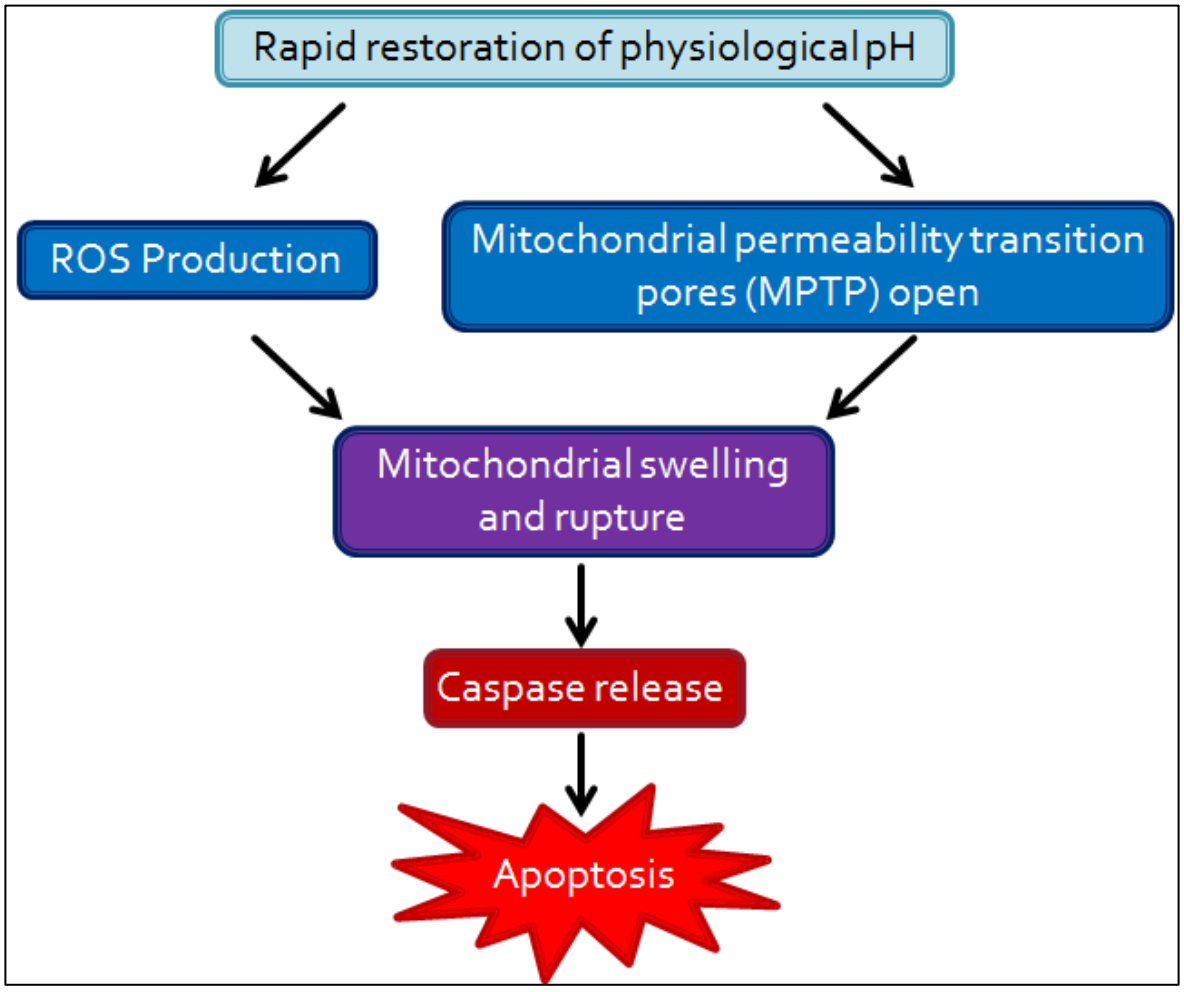

Figure 2. Pathophysiology of reperfusion injury. Chain of events that occur during reperfusion injury, ultimately resulting in myocardial apoptosis.

Apoptotic myocardium is most commonly found at the periphery of the infarct region, as the vast majority of the center is comprised of necrotic tissue. The duration of MPTP opening is variable among cells experiencing reperfusion, and plays an integral role in the determination of infarct size.[16,17] As the pores remain open, oxidative phosphorylation becomes unavailable to the mitochondria, and the F_1F_0 begins to function in reverse; ATP synthesis reverses into hydrolysis. If the pores are unable to close before too much ATP is expended, the myocyte will be unable to perform apoptosis and will, instead, become necrotic, causing severe damage to the myocardium.[7]

Additionally, rapid reperfusion brings with it a flood of reactive oxygen species (ROS) produced by the freshly fueled ETC, as well as endothelial xanthine oxidase, large doses of nitric oxide, and NADPH oxidase secreted by neutrophils recruited by MPTP opening. The ROS serve to cause additional damage to membranes, as well as damage the cell's sarcoplasmic reticulum. These ruptures in the SR cause an additional spike in $[Ca^{+2}]_i$, causing further mitochondrial swelling, and myofibril hypercontracture.[7,16,17] Despite these challenges, reperfusion remains the best treatment method available for ischemic heart injury. It's mechanisms are complex, possessing an intricate series of events that beckon for additional scientific refinement. What is certain, however, is that all of these mechanisms converge on the fate of the mitochondria, an organelle that is highly susceptible to manipulation of metabolic regulatory systems.

1.4 Mammalian Target of Rapamycin (mTOR)

Mammalian Target of Rapamycin (mTOR) is a Serine-Threonine kinase whose primary function is to act as an intracellular sensor to environmental energy and nutrient cues. Responding to these signals, mTOR catalyzes cellular transitions between catabolic and anabolic states, thereby controlling cell growth and metabolism.[18-22] By relying on nutrient-based signaling, altered metabolic states, such as diabetes mellitus, have a tendency to misregulate mTOR and cause cellular dysfunction. Likewise, mTOR pathway malfunctions frequently lead to metabolic diseases, such as Type II Diabetes. It has garnered a great deal of interest due to its multifaceted role in a variety of fields such as metabolic stress, aging, oncology, neurodegeneration, and cardiovascular disease (Figure 3).[19-25] Discoveries have shown that the mTOR protein interacts with a collection of proteins to form two distinct complexes: the aptly named mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) (Figure 4).[22,26,27]

Central to both complexes is mTOR, a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family.[28] Additionally, both complexes also contain DEPTOR (DEP domain containing mTOR-interacting protein), the Tti1-Tel2 complex, and the mLST8 (mammalian lethal with sec-13 protein 8) catalytic mTOR subunit. Unique to mTORC1 are Raptor (regulatory-associated protein of mTOR) and PRAS40 (proline-rich Akt Substrate), whereas mTORC2 contains Rictor (rapamycin-insensitive companion of mTOR) and mSin1 (mammalian stress-activated MAP kinase-interacting protein 1).[21,26]

mTORC1 responds to the presence of nutrients and amino acids, activating the Rheb GTPase that is responsible for releasing PRAS40 inhibition from the Raptor

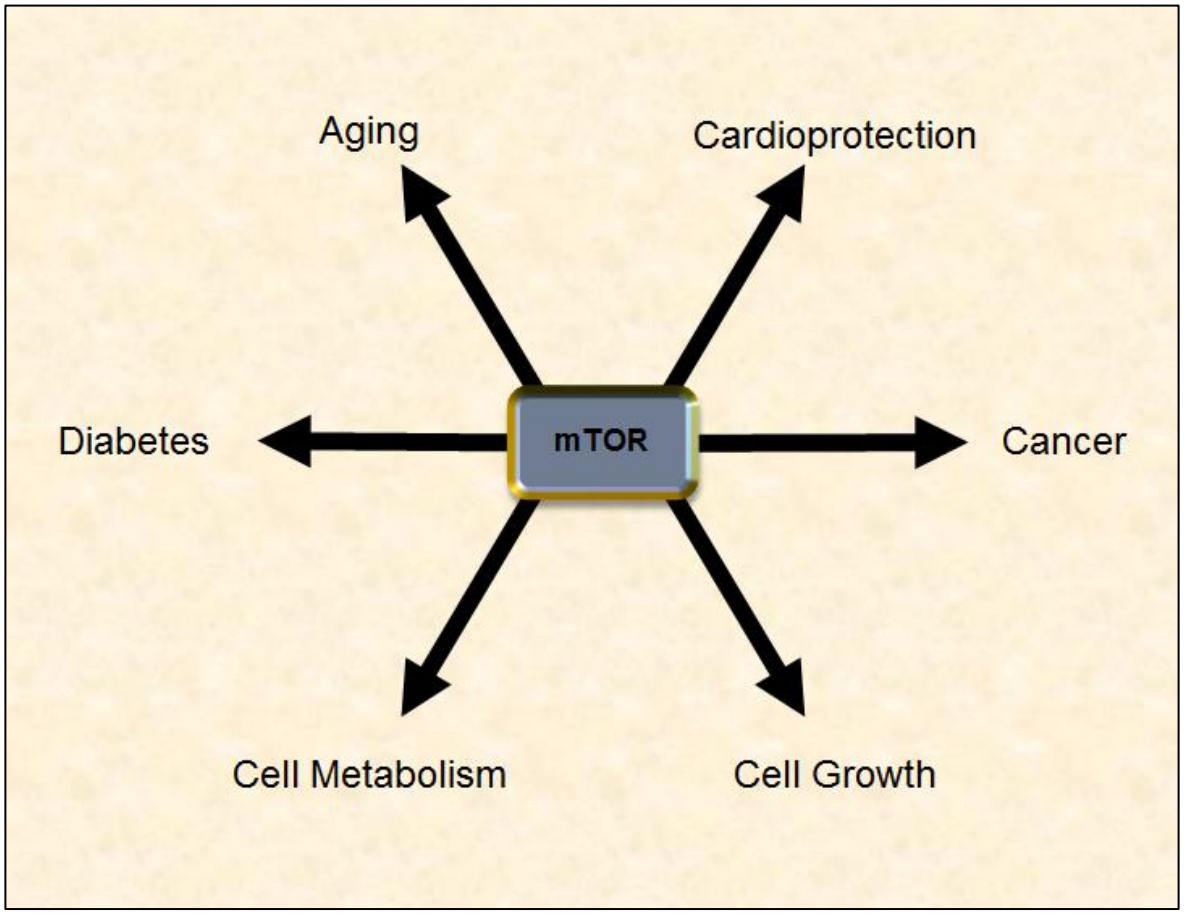

Figure 3. mTOR relevance across multiple fields of study. The mTOR protein has been found to play a significant role in a myriad of different fields.

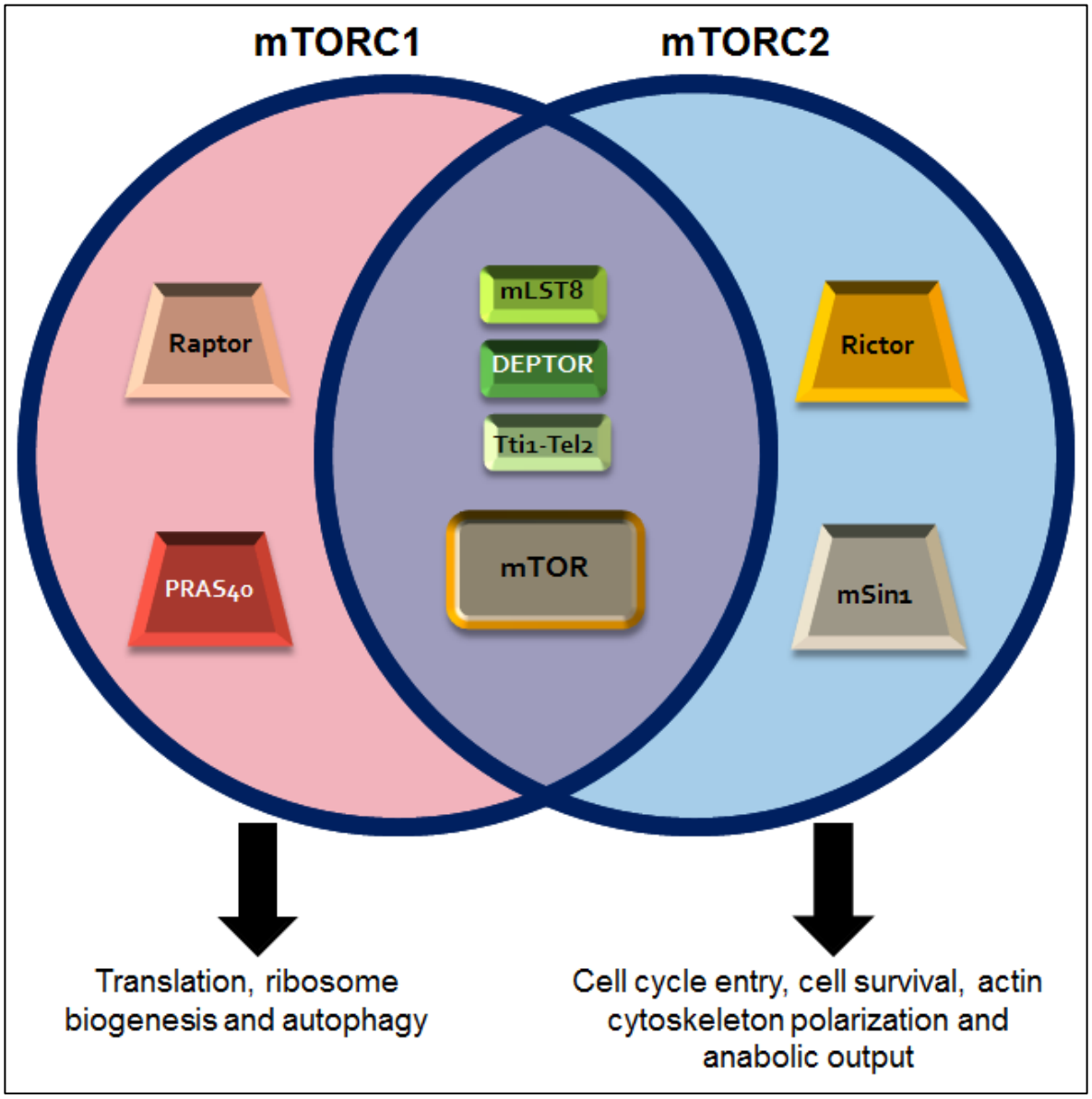

Figure 4. Structural differences between mTORC1 and mTORC2. Venn diagram illustrating both the common and unique components of mTORC1 and mTORC2. Both complexes contain the mTOR protein, as well as mLST8, DEPTOR, and the Tti-Tel2 complex. Additionally, mTORC1 contains the Raptor and PRAS40 subunits while mTORC2 is bound to Rictor and mSin1.

subunit.[29,30] It promotes translation, ribosome biogenesis, and autophagy by regulating two major downstream targets: The eIF4E-binding protein 1 (4FBP1) and ribosomal P70S6K (S6).[31] 4FBP1 controls cap-dependent translation initiation whereas S6 oversees elongation; their activation occurs by phosphorylation. Furthermore, S6 is a potent inhibitor of autophagy, and an excellent measure of mTORC1 function.[21,26,32]

mTORC2 is respondent to growth factors, promoting cell-cycle entry, cell survival, actin cytoskeleton polarization and anabolic synthesis.[26,33-35] Downstream targets of mTORC2 are the Ser-Thr kinases Akt, PKC, and SGK. Phosphorylated at Ser^{473}, Akt is a major component of the mTOR cell survival cycle, responsible for downstream inhibition of Beclin-1 mediated autophagy, crosstalk with mTORC1, and anti-apoptotic Bcl-2 induction/Bax inhibition in conjunction with abolishing p53.[36] Selective manipulation of the mTOR pathway has yielded promising results in the search for more effective methods of cardioprotection (Figure 5). Rapamycin is one such method, the original antagonist from which TOR's name was derived.

1.5 Role of mTOR in I/R injury

mTOR is responsible for regulating the cellular response to conditions of energy deprivation and ischemia.[37] During these stressful situations, mTORC1 is frequently inhibited in order to promote cell survival. Partial inhibition of mTORC1 activates cellular autophagy, while also maintaining energy reserves by scaling down energy-depleting

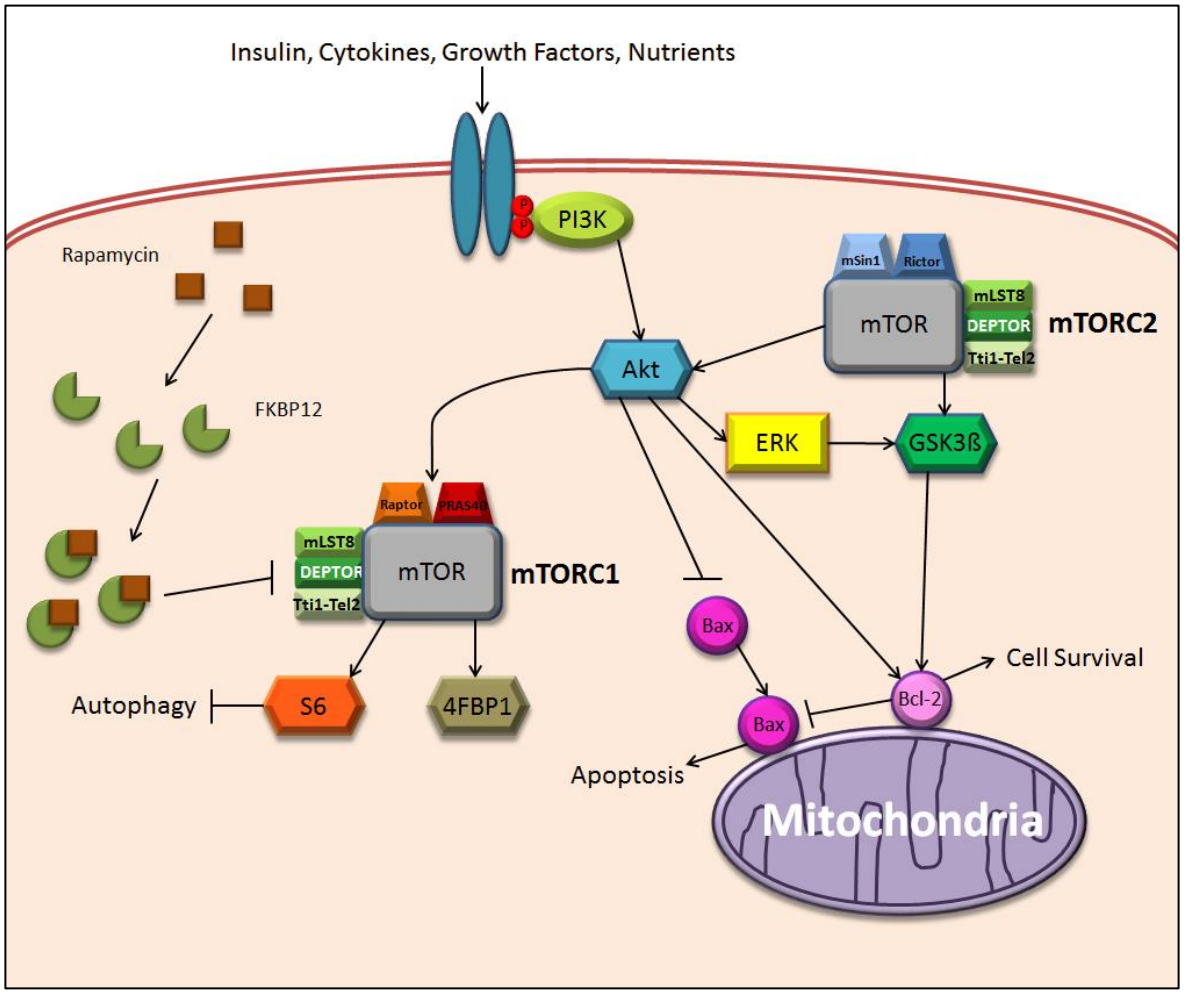

Figure 5. mTOR signaling pathways. Schematic detailing mechanisms of the PI3K-Akt-mTOR signaling pathway and how it is affected by Rapamycin.

metabolic processes.[17,25,38,39] It has been demonstrated that Rheb-dependent mTORC1 inhibition plays a major role in cardioprotection.[40] Downstream inhibition of S6K allows for autophagy activation and inhibition of protein synthesis, reducing ischemic damage and preserving cardiac function.

Conversely, mTORC2 inhibition increases infarct size in the ischemic heart.[41] In fact, activation of mTORC2 has been proven to yield cardioprotective results via Akt-mediated cell survival mechanisms. While overexpression of mTORC2 serves to reduce infarct size, genetic inhibition of Akt has shown some preserved cardioprotective function. Thus, it has been inferred that mTORC2 cardioprotection operates via both Akt-dependent and –independent mechanisms.[42,43] mTOR inhibition with rapamycin induces potent preconditioning-like effects against myocardial I/R injury by limiting infarct size and cardiomyocyte necrosis and apoptosis.[23] Furthermore, the immunosuppressant drug Rapamycin has been shown to have cardioprotective properties by inhibiting mTORC1 function and inducing optimum levels of autophagy (Figure 6).[44]

1.6 Rapamycin

Rapamycin (Sirolimus®) is an anti-fungal macrolide derived from the *Streptomyces hygroscopicus* bacterial strain. Originally isolated from a soil sample from the Eastern Islands in the 1970s, the drug proved to be a valuable research tool in studying cell growth and proliferation.[18] Technological advances in the 1990s made way for the discovery of Rapamycin's action on the mTOR complex in yeast and, shortly thereafter, mammals.[45-49]

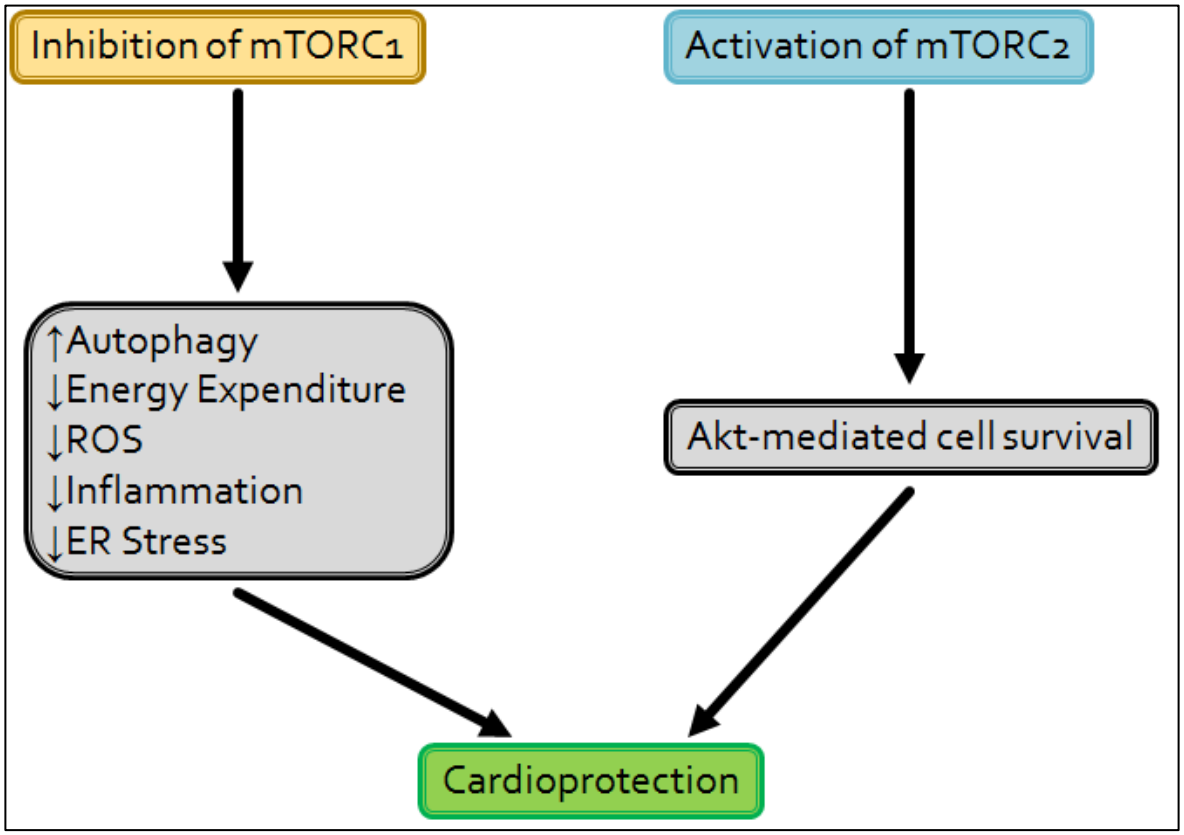

Figure 6. mTOR-mediated cardioprotection. Both selective inhibition of mTORC1 and the activation of mTORC2 yield cardioprotective benefits.

It displays a remarkable ability to halt cell growth and has been adopted as an effective immunosuppressant used to coat post-angioplasty drug-eluting stents (to reduce the risk of restenosis), as well as prevent rejection of coronary artery grafts and organ transplants.[50-53]

Rapamycin interferes with mTOR function by the blockade of the crowded ATP-binding catalytic domain of the mTOR complex. Flanked by a large N- and C-terminal lobe on either side, this hydrophobic active site lies within a deep valley and is responsible for drawing in and phosphorylating mTOR's downstream substrates.[22] In the presence of the drug, Rapamycin's direct binding target, FK506-binding protein 12 (FKBP12) forms a ternary complex with the FRB domain of mTOR.[6,37] The end result is catalytic domain occlusion, and allosteric inhibition of MTORC1; mTORC2 function is not affected by normal doses of Rapamycin.[22,24,26,31] Recent studies have shown the effectiveness of ATP-competitive inhibitors, such as the anticancer agent Torin2, which inhibits both mTORC1 and mTORC2.[38,53] These inhibitors have assisted in understanding the delicate balance involved between the two mTOR complexes their complex signaling networks. mTOR participates in crosstalk between both the PI3K-Akt and MAPK signal transduction pathways.[16,17]

In 2006, the cardioprotective effect of rapamycin against I/R injury in isolated mouse heart and cardiomyocytes was first reported.[23] Attenuation of I/R injury with rapamycin is mediated through opening of mitochondrial K_{ATP} channels (ATP-sensitive potassium channel). Soon thereafter, further signaling mechanisms in rapamycin-induced

cardioprotection were identified. Rapamycin triggered unique cardioprotective against I/R injury signaling through JAK2-STAT3 signaling.[54] This study showed the ERK-dependent phosphorylation of STAT3, which is causatively involved in reducing I/R injury in the heart and cardiomyocytes. Rapamycin also causes a significant increase in phosphorylation of GSK3β at Ser9, inactivating the protein.[55] It has been shown that activation of GSK3β induces myocardial apoptosis by increasing mitochondrial depolarization and releasing cytochrome C and eventual cell death. Inhibition of GSK3β activation induces cardioprotection during I/R by regulating the MPTP opening through direct phosphorylation.

Pro-apoptotic proteins, such as Bax, and anti-apoptotic proteins, including Bcl-2, are responsible for MPTP regulation in situations of cellular stress, tipping the balance between cell death or survival. Rapamycin enhances the prosurvival Bcl-2 to Bax ratio by inactivating GSK3β, which may mediate rapamycin-induced cardioprotection against I/R injury.[54]

The major goal of this study was to further elucidate the role of Rapamycin, in conjunction with reperfusion therapy and to understand the associated signaling pathways with cardioprotection. We believe that these studies will help us in future translation of the concept of mTOR inhibition in cardioprotection in the clinical setting.

Materials and Methods

2.1 Chemicals and Reagents

Rapamycin (Sirolimus®) was purchased from LC Laboratories (MA). ApoAlert™ DNA Fragmentation Assay kit was purchased from BD Biosciences, Palo Alto, CA. DAPI was purchased from Vector Laboratories, Inc. CA. Antibodies for phospho-serine 473-Akt, Akt, p-S6, S6, phospho-tyrosine 705-STAT3, STAT3, p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2, p-P38, P38, Bcl-2, and Bax were purchased from Cell Signaling Technology and GAPDH-HRP was purchased from Santa Cruz Biotechnology.

2.2 Animals

Adult C57BL/6J mice (body weight ~30 g) were supplied by Jackson Laboratories. The animal care and experiments were approved by the Institutional Care and Use Committee of Virginia Commonwealth University.

2.3 Experimental Groups

For *in vivo* regional I/R protocol, 3 groups were used: 1) DMSO without I/R, 2) DMSO with I/R, or 3) Rapamycin (0.25 mg/kg) with I/R. DMSO/Rapamycin was injected (intracardial) immediately at the onset of reperfusion following 30 min of *in situ* ischemia (Figure 7).

2.4 Myocardial Infarction Protocol

The *in vivo* myocardial I/R procedures were conducted in mouse by ligation of the left anterior descending coronary artery (LAD) according to a previously reported method.[19] To simulate ischemia, animals were anesthetized with pentobarbital sodium (70 mg/kg, i.p.), and ventilated on a positive pressure ventilator. A left thoracotomy was performed at the fourth intercostal space, and the heart was exposed by stripping the pericardium. The LAD was occluded for 30 min by a 7.0 silk ligature that was placed around it and a small piece of polyethylene tubing (PE10) that was positioned on top of it. Reperfusion was established by removing the PE10 tube that was compressing the LAD. Just prior to reperfusion, a bolus of Rapamycin (0.25 mg/kg, i.c.) or DMSO (0.2 mL, i.c.) was injected intracardially (Figure 7). Air was then expelled from the chest, and the chest cavity was closed. The animal was placed into a cage on a heating pad until fully conscious, and left to reperfuse for 24 hours.

2.5 Measurement of Infarct Size

The heart was removed following 30 min of ischemia and 24 hr of reperfusion, and mounted on a Langendorff apparatus. The coronary arteries were perfused with 37°C Krebs-Henseleit buffer. After the blood was washed out, 3 ml of 10% TTC in isotonic phosphate buffer (pH 7.4) at 37°C were infused over several minutes before the ligature

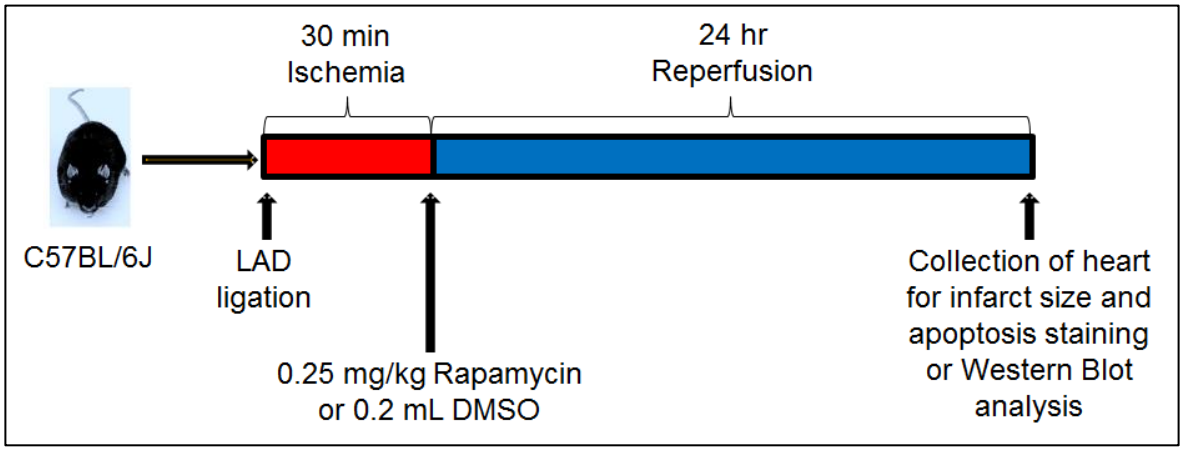

Figure 7. Experimental design. Experimental groups and protocol of regional I/R by LAD occlusion in C57 mouse hearts.

was retightened and ~1 ml of 5% Phthalo blue dye was injected as a bolus into the aorta until most of the heart turned blue. The heart was perfused with saline to wash out the from apex to base of equal thickness (~1 mm). The slices were then fixed in 10% neutral buffered formaldehyde for 4 to 24 h with a weight on top to keep the heart slices flat for the initial 30 min. The areas of infarcted tissue, the risk zone, and the whole left ventricle were determined by computer morphometry using ImageJ imaging software.

2.6 Apoptosis Assay

Apoptosis was determined by TUNEL staining using an ApoAlert^{TM} DNA Fragmentation Assay kit purchased from BD Biosciences according to previously reported protocol.[56] Hearts were stored in a 10% formalin solution, and paraffin–embedded tissue section was mounted on glass slides. Apoptosis was then assessed in the transverse sections of paraffin sections. Sections were deparaffinized by immersing slides in fresh xylenes. The slides were then immersed in 96% ethanol, 90% ethanol and 80% ethanol for 5 min each. The sections were rehydrated by immersing in distilled water for 3-5 min which was followed by immersion in citrate buffer (pH 6.0) and boiled for 10 min. Slides were then incubated with nucleotide mix, TdT enzyme in equilibration buffer according to manufacturer's protocol (BD Biosciences, CA) for 1 hr at 37^0C under dark. The reaction was terminated by immersing slides in 2XSSC buffer for 15 min. After three wash cycles with PBS (phosphate saline buffer), tissue sections on slides were incubated in mounting medium for fluorescence with DAPI (Vector Laboratories, Inc. CA). The sections were

then covered with a glass coverslip. Apoptotic cells were examined under a fluorescence microscope (Nikon Eclipse T*i*) which were clearly identified with a strong nuclear green fluorescence. All cell nuclei were visualized as blue fluorescence following staining with DAPI. The apoptotic index was expressed as the number of apoptotic cells of all cardiomyocytes per field (n=3). Apoptotic rate in the peri-infarct regions was calculated using 6 random fields, which is primarily composed of the peri-infarct area.[56]

2.7 Western Blots

Total soluble protein was extracted from the whole heart tissue with extraction buffer. Seventy-five (75 ug) protein from each sample was separated by SDS-PAGE and transferred onto nitrocellulose membrane (0.2 um pore size). The membrane was incubated in cold conditions overnight with primary antibodies (phospho-serine 473-Akt, Akt, p-S6, S6, phospho-tyrosine 705-STAT3, STAT3, p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2, p-P38, P38, Bcl-2, and Bax from Cell Signaling Technology; GAPDH-HRP from Santa Cruz Biotechnology) in a 1:1000 dilution with 5% BSA. The membrane was washed and incubated with horseradish peroxidase conjugated secondary antibody (1:3000 dilution in 5% milk solution) and the blots were developed using a chemiluminescent system (Western Lighting Plus-ECL; Perkin Elmer, Inc.).

2.8 Data Analysis and Statistics

Data are presented as mean±SEM. The differences between groups were analyzed with one-way analysis of variance followed by Student-Newman-Keuls post hoc test for pairwise comparison, or by unpaired t-test. $P<0.05$ was considered to be statistically significant. All statistical analyses were performed using Prism statistical software.

Results

3.1 Rapamycin reduces infarct size

Rapamycin treatment at the onset of reperfusion resulted in a significant reduction in infarct size (% of risk area) to a mean of 28.2±1.2 as compared to 46.1±2.7 in the DMSO-treated control group ($n=4$, $p<0.01$) after 30 min ischemia and 24 hour reperfusion (Figure 3B). Total risk area was not statistically different between Rapamycin (60.0%±2.3) and DMSO (54.5%±1.2) treated groups ($n=4$, $p>0.05$) following *in vivo* I/R (Figure 8A).

3.2 Effect of Rapamycin on Myocardial Apoptosis

Tissue sections from Rapamycin treated hearts showed a significant reduction in TUNEL-positive nuclei in the peri-infarct regions. The Rapamycin group decreased to 3.4±1.0% as compared to 10.0±1.3% in the DMSO-treated group ($n=3$, $p<0.05$) (Figure 9A and B).

3.3 Rapamycin inhibits mTORC1 and promotes mTORC2 activity

To further elucidate the effects of Rapamycin on mTOR, S6 and Akt phosphorylation levels were analyzed by Western Blot to gauge mTORC1 and mTORC2 activity, respectively. S6 phosphorylation at Ser 235/236 displayed an increasing trend among samples in the DMSO-treated I/R group, compared to control (no I/R) C57 mouse

hearts. This phosphorylation was significantly reduced with Rapamycin treatment, displaying no major change relative to the control group (Figure 10, n=3, $p<0.05$). *In vivo* I/R elicited no major change among Akt Ser 473 phosphorylation compared to control. However, post-ischemic Rapamycin treatment significantly increased phosphorylation at this site (Figure 11, n=3, $p<0.01$).

3.4 Ischemia/reperfusion Increases STAT3 Phosphorylation

STAT3 phosphorylation was increased in both DMSO- and Rapamycin-treated I/R groups compared to C57 control hearts (n=3, $p<0.05$), with no significant changes between the I/R groups (Figure 12A). Additionally, total STAT3 protein levels were decreased following I/R, and restored with Rapamycin treatment (n=3, $p<0.05$)(Figure 12B).

3.5 Effect of Rapamycin on MAP Kinase Signal Transduction

To analyze the effect of post-ischemic Rapamycin treatment on the MAP Kinase signaling pathway, phosphorylation levels of ERK1/2, JNK1/2, and P38 were measured using western blot analysis. Both pERK1 and pERK2 were significantly increased following post-ischemic Rapamycin treatment compared to the control and DMSO-treated I/R groups (n=3, $p<0.01$)(Figure 8A, B, and C). Total ERK1 and 2 levels were not affected among any of the treatment groups (n=3, $p>0.05$)(Figure 13A, B, and C). Phospho-P38, as well as total P38, levels remained unchanged between control and I/R DMSO groups (n=3, $p>0.05$), but significantly decreased with Rapamycin treatment (n=3, $p<0.01$)(Figure 14).

While p-JNK1 and 2 did not display statistically significant changes, results showed a substantial positive trend with *in vivo* I/R, and a further increase following Rapamycin treatment ($n=3$, $p>0.05$)(Figure 15A, B, and C). Total JNK1,2 and JNK1 levels increased following *in vivo* I/R, compared to the control; Rapamycin did not appear to alter this elevated state of expression ($n=3$, $p<0.05$)(Figure 15E and F). Total JNK2, alone, yielded a similar trend, but results were not statistically significant ($n=3$, $p>0.05$)(Figure 15G).

3.6 Rapamycin Blocks Pro-Apoptotic Bax Signaling

Bcl-2 and Bax levels were measured to identify pro-survival and pro-apoptotic cell signaling, respectively. Bcl-2 levels decreased following I/R, which showed only a positive increasing trend following Rapamycin treatment ($n=3$, $p<0.05$)(Figure 16A). Pro-apoptotic Bax was significantly increased following *in vivo* I/R ($n=3$, $p<0.05$), but its expression was reduced to a pre-ischemic state with post-ischemic Rapamycin administration ($n=3$, $p<0.01$)(Figure 16A). The Bcl-2/Bax ratio showed significant decline in both I/R groups ($n=3$, $p<0.05$); however, there was an increasing trend in the Rapamycin treatment group (Figure 16B).

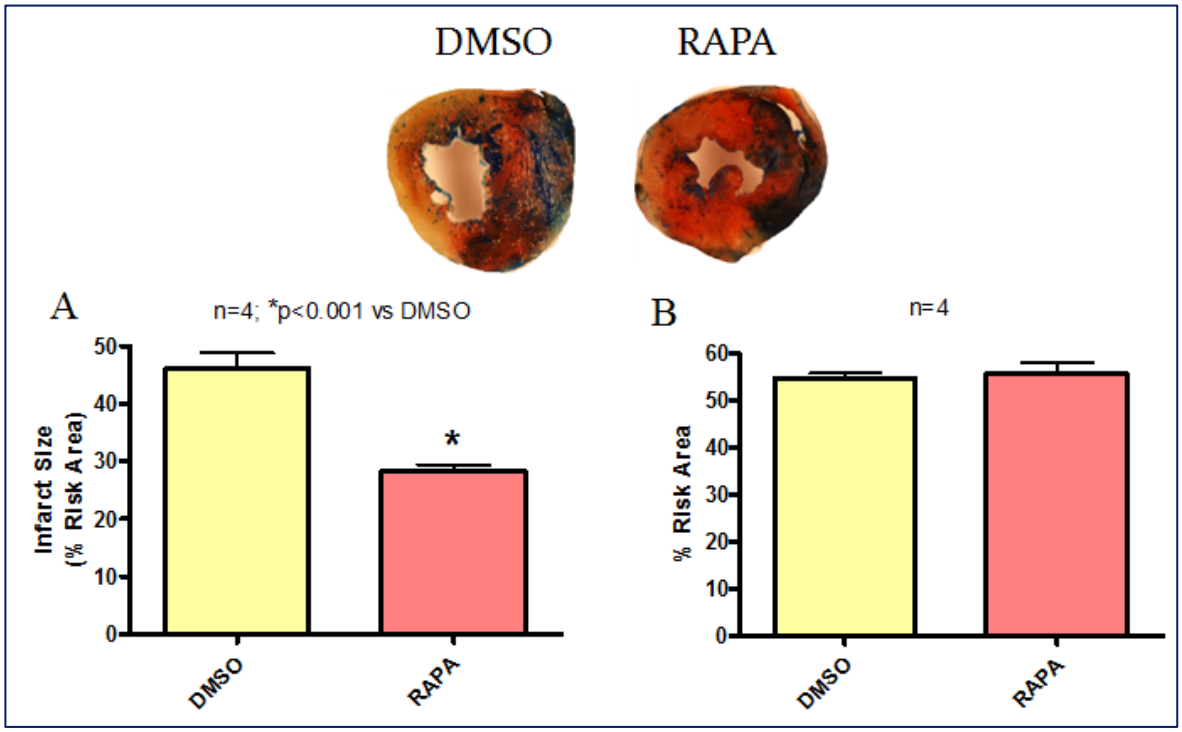

Figure 8. Effect of Rapamycin on infarct size. (A) Infarct region was determined by tetrazolium chloride (TTC) assay after *in vivo* I/R. Infarct size was then calculated as a % of the total measured risk area. *p<0.01 vs DMSO, n=4 (B) Risk area measurement, obtained via Evans Blue assay, as % of total tissue sample, n=4.

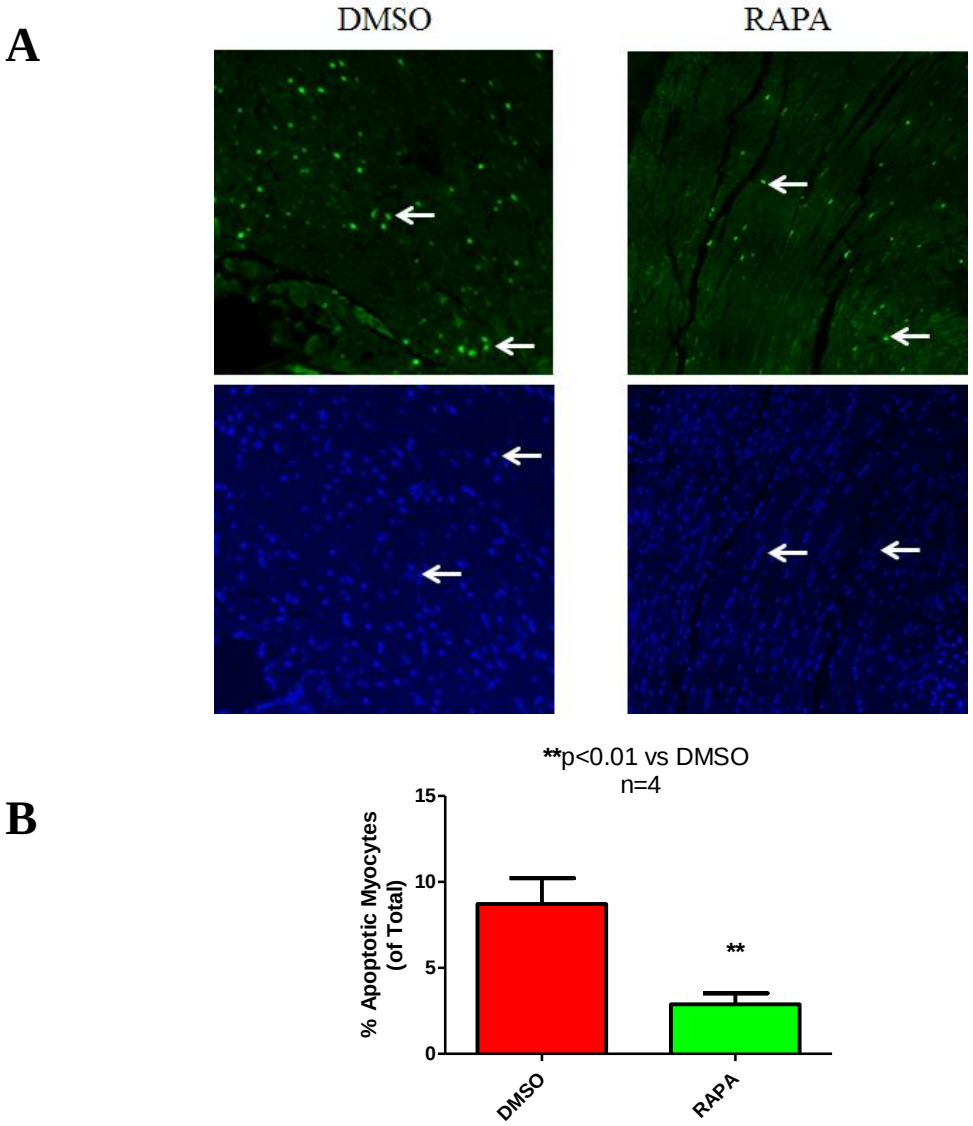

Figure 9. Effect of Rapamycin treatment on myocardial apoptosis. Myocardial apoptosis was determined by TUNEL assay following *in vivo* I/R. (A) Representative images of TUNEL-positive nuclei in bright green fluorescent color (White arrows) and total nuclei staining with 4,5-diamino-2-phenylindole (DAPI). (B) Bar graph showing quantitative data of TUNEL positive nuclei in myocardium. *p<0.05 vs DMSO, n=3 per group. Refer to Fig. 1 for drug treatment schedules and I/R protocol.

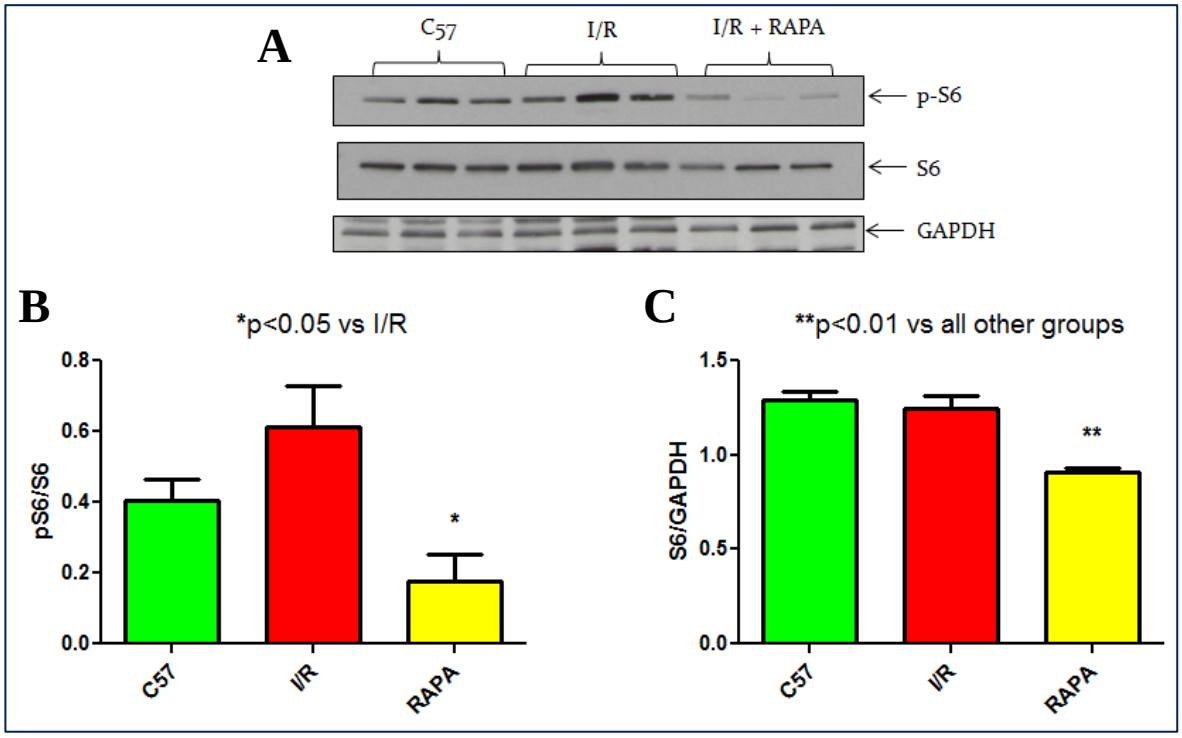

Figure 10. Rapamycin (RAPA) attenuates phosphorylation of S6 Ser235/236. (A) Representative immunoblots for p-S6, total S6, and GAPDH expression in whole heart tissue samples after *in vivo* I/R (no I/R in C57 control). (B) Densitometry analysis of immunoblots for the ratio of p-S6/S6. *p<0.05 vs I/R, n=3. (C) Densitometry analysis of immunoblots for the ratio of S6/GAPDH,**p<0.01 vs all other groups, n=3.

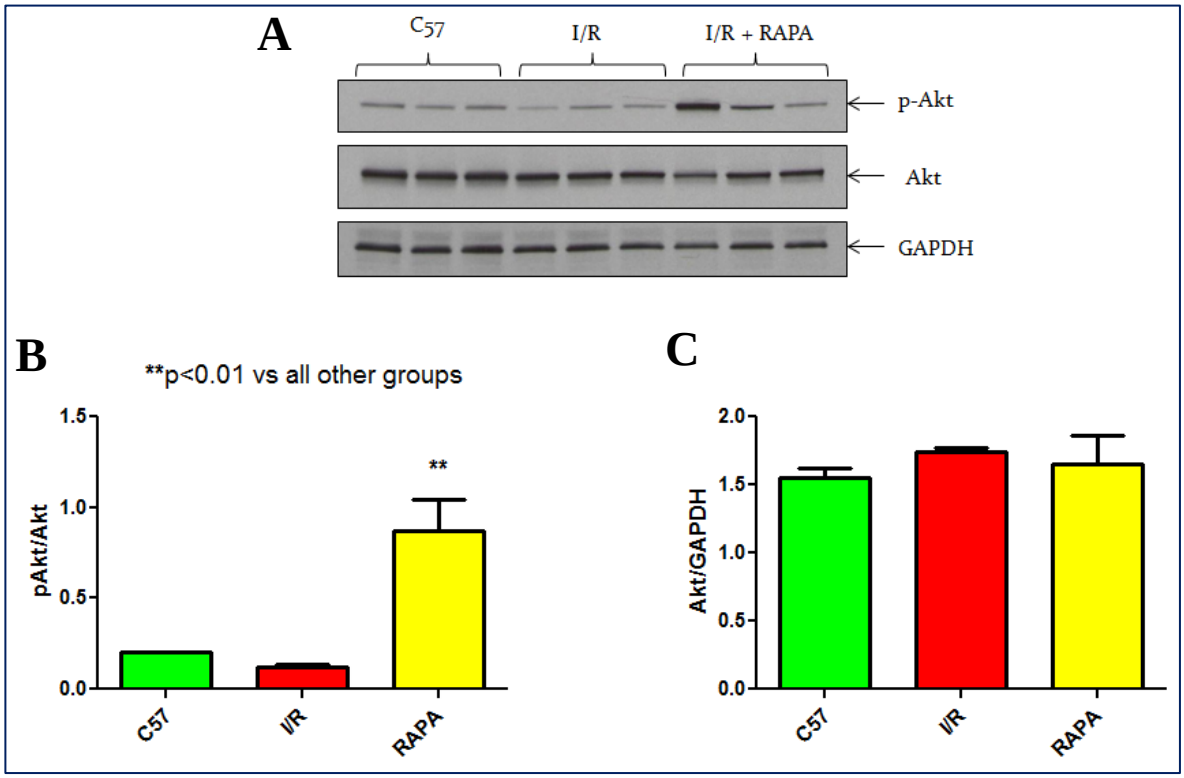

Figure 11. Rapamycin (RAPA) enhances phosphorylation of Akt Ser473. (A) Representative immunoblots for p-Akt, total Akt, and GAPDH expression in whole heart tissue samples after *in vivo* I/R (no I/R in C57 control). (B) Densitometry analysis of immunoblots for the ratio of p-Akt/Akt. **p<0.01 vs all other groups, n=3. (C) Densitometry analysis of immunoblots for the ratio of Akt/GAPDH, n=3.

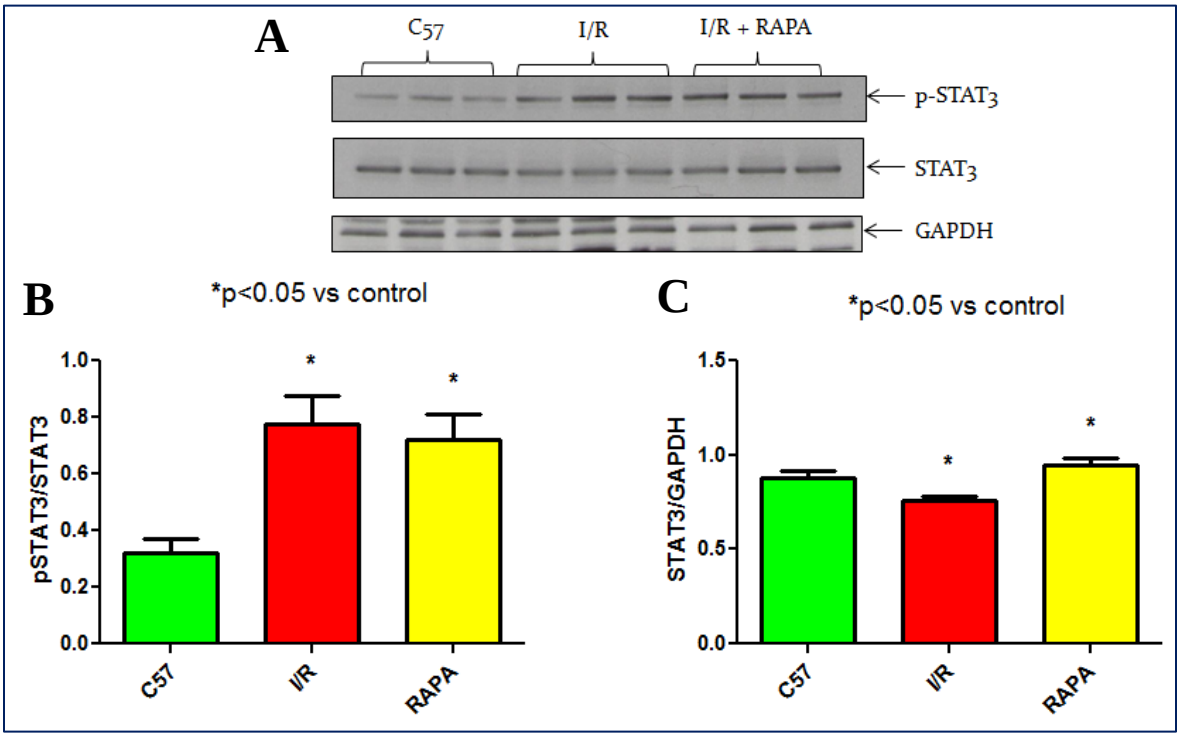

Figure 12. ***In vivo*** **I/R induces STAT3 phosphorylation while Rapamycin shows no significant changes**. (A) Representative immunoblots for p-STAT3, total STAT3, and GAPDH expression in whole heart tissue samples after *in vivo* I/R (no I/R in C57 control). (B) Densitometry analysis of immunoblots for the ratio of p-STAT3/STAT3. *p<0.05 vs C57 control, n=3. (C) Densitometry analysis of immunoblots for the ratio of STAT3/GAPDH. *p<0.05 vs C57 control, n=3.

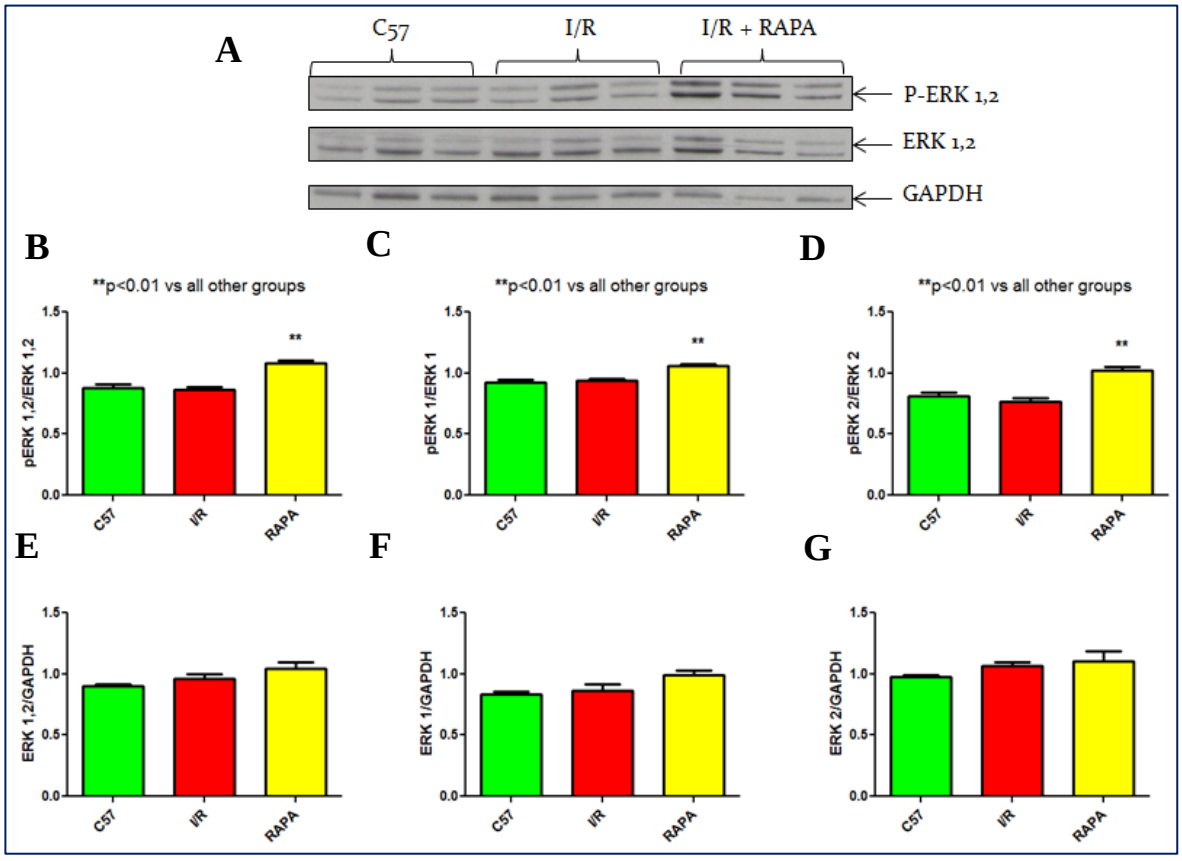

Figure 13. Rapamycin (RAPA) induces ERK phosphorylation following I/R. (A) Representative immunoblots for p-ERK1,2, p-ERK1, p-ERK2, total ERK1,2, ERK1, ERK2, and GAPDH expression in whole heart tissue samples after *in vivo* I/R (no I/R in C57 control). (B) Densitometry analysis of immunoblots for the ratio of p-ERK1,2/ERK1,2. **p<0.01 vs all other groups, n=3. (C) Densitometry analysis of immunoblots for the ratio of pERK1/ERK1, **p<0.01 vs all other groups, n=3. (D) Densitometry analysis of immunoblots for the ratio of pERK2/ERK2,**p<0.01 vs all other groups, n=3. (E)(F)(G) Densitometry analysis of immunoblots for the ratio of total ERK variants relative to GAPDH, n=3.

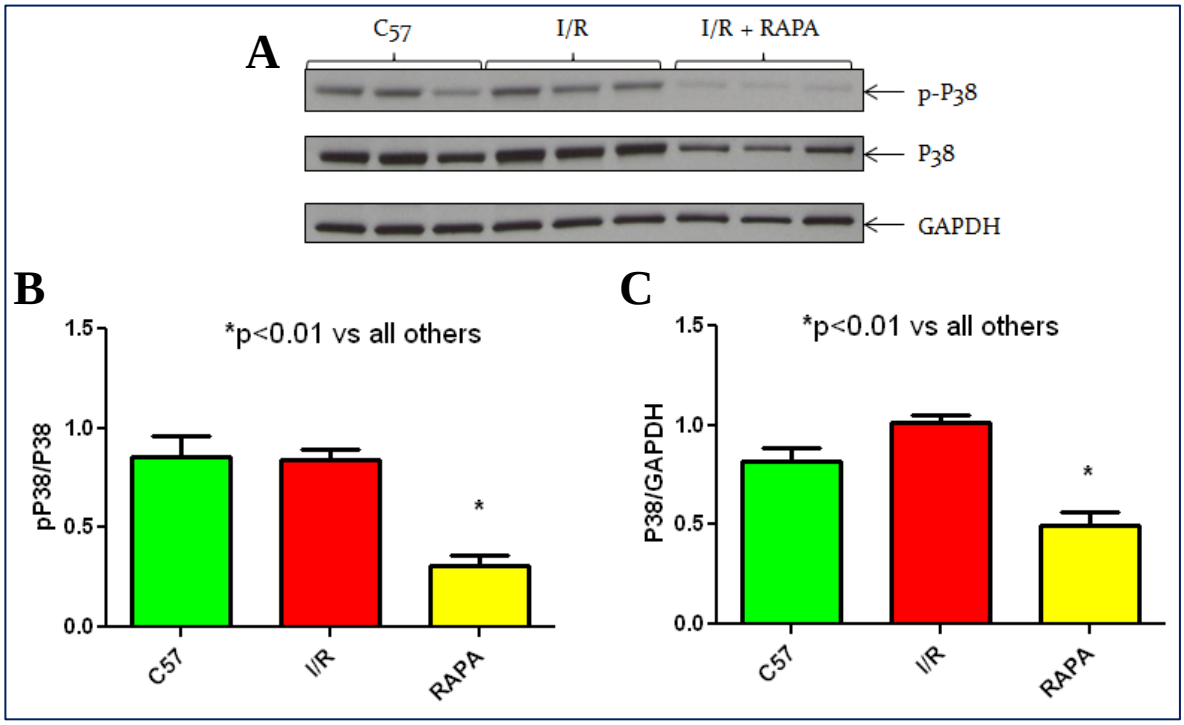

Figure 14. Rapamycin (RAPA) decreases P38. (A) Representative immunoblots for p-P38, total P38, and GAPDH expression in whole heart tissue samples after *in vivo* I/R (no I/R in C57 control). (B) Densitometry analysis of immunoblots for the ratio of p-P38/P38. *p<0.01 vs all other groups, n=3. (C) Densitometry analysis of immunoblots for the ratio of S6/GAPDH,*p<0.01 vs all other groups, n=3.

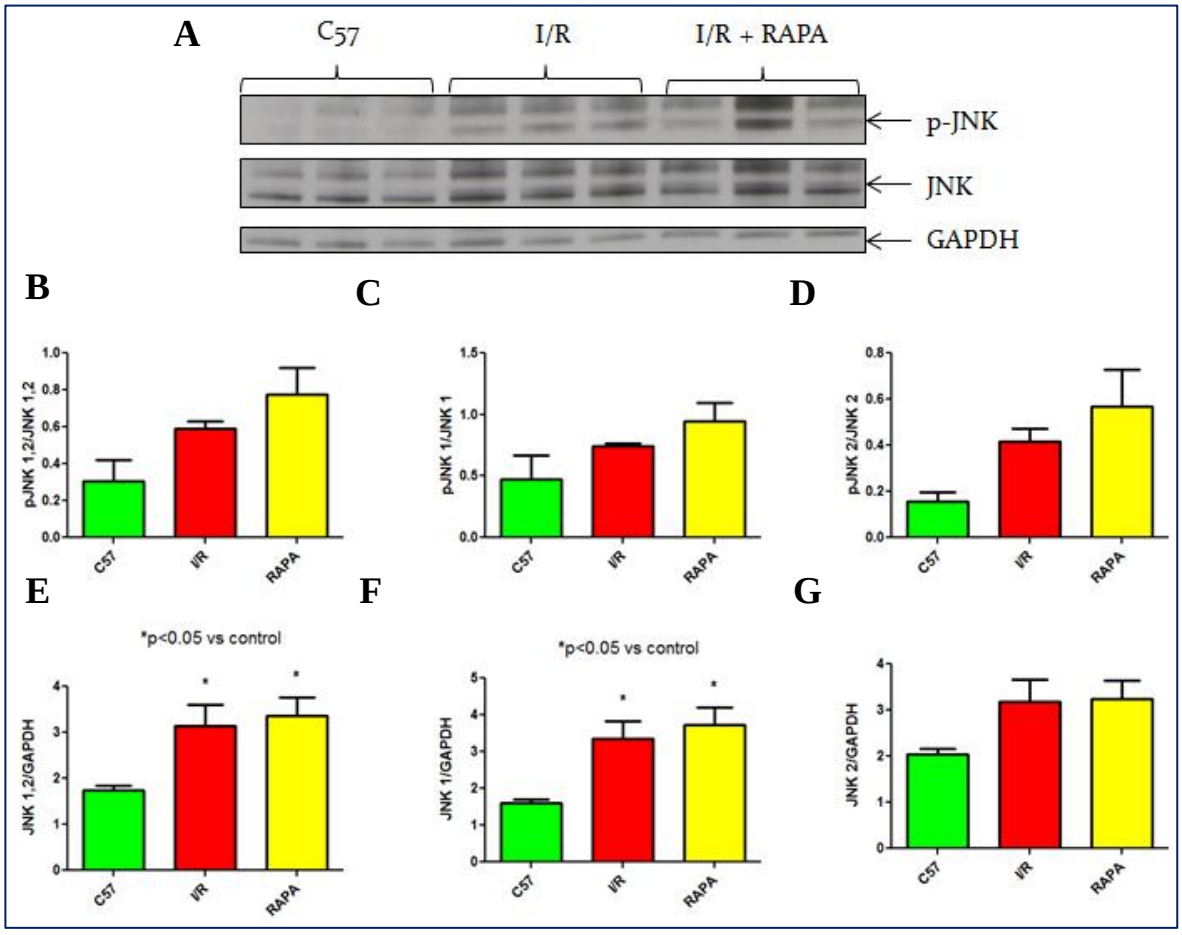

Figure 15. Ischemia/reperfusion (I/R) increases total JNK – modulation of its phosphorylation by Rapamycin. (A) Representative immunoblots for p-JNK1,2, p-JNK1, p-JNK2, total JNK1,2, JNK1, JNK2, and GAPDH expression in whole heart tissue samples after *in vivo* I/R (no I/R in C57 control). (B)(C)(D) Densitometry analysis of immunoblots for the ratio of JNK phosphorylation compared to total protein levels, n=3. (E)(F)(G) Densitometry analysis of immunoblots for the ratio of total ERK variants relative to GAPDH. *p<0.05 vs C57 control, n=3.

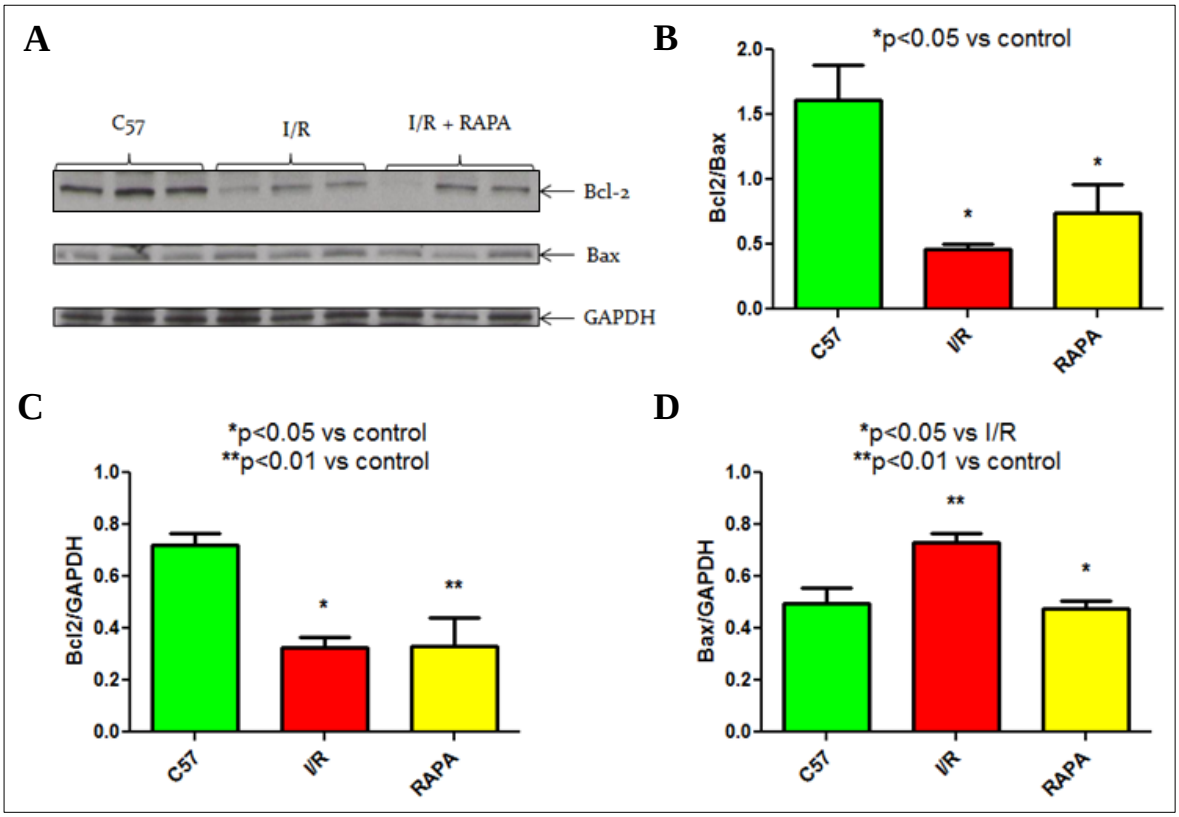

Figure 16. Rapamycin (RAPA) decreases Bax levels in adult C57 mouse hearts. (A) Representative immunoblots for Bcl-2, Bax, and GAPDH expression in whole heart tissue samples after *in vivo* ischemia/reperfusion (I/R); (no I/R in C57 control). (B) Densitometry analysis of immunoblots for the Bcl-2/Bax ratio. *p<0.01 vs C57 control, n=3. (C) Densitometry analysis of immunoblots for the ratio of Bcl2/GAPDH,*p<0.01 vs control, **p<0.01 vs control, n=3. (D) Densitometry analysis of immunoblots for the ratio of Bax/GAPDH,**p<0.01 vs control, *p<0.05 vs I/R, n=3.

Discussion

Cardiovascular disease is the current leading cause of death among the Western nations. In 2007, almost 8 million individuals were affected by acute myocardial infarction (AMI).[57] Clinically the goal is to re-establish blood flow to the ischemic area as quickly as possible to salvage cardiomyocytes that would be damaged by ischemia. While reperfusion is necessary for tissue survival, it is worth noting that reperfusion itself can also cause tissue damage, termed "reperfusion injury". As more tissue is irreversibly injured, the prognosis becomes worse because terminally differentiated cardiac myocytes cannot regenerate. Loss of contractile mass puts an inordinate load on surviving tissue thereby causing the remaining cells to hypertrophy resulting in adverse remodeling of the ventricle ending ultimately in heart failure. Therefore, strategies that would render heart cells resistant to death from ischemia/reperfusion injury would greatly improve the prognosis of AMI.

In the present study, we investigated the cardioprotective mechanisms during post-ischemic Rapamycin treatment in adult mouse hearts. Furthermore, we interrogated the mTOR complex, and it's involvement with downstream PI3K-Akt and MAPK signaling pathways due to their role in cell survival. Rapamycin, an mTOR antagonist, has drawn attention from it's ability to selectively inhibit mTORC1, making it a potential therapeutic agent.[7] Activation of anti-apoptotic proteins has been shown to play an integral role in

cardioprotection, and pre-treatment with Rapamycin has been shown to reduce both the infarct size in the heart and cardiomyocyte apoptosis following simulated ischemia and reperfusion.[19,23,54,56] However, in direct contrast to studies outlining it's benefits, Rapamycin has been shown to abolish the cardioprotective effects of pharmacological preconditioning, by increasing infarct size.[19,23,54,56] The present study demonstrates Rapamycin-induced cardioprotection *in vivo*, as evidenced by decrease in myocardial infarct size and reduced level of apoptosis in the heart. We believe that these *in vivo* studies are highly relevant for potential clinical translation of cardioprotective Rapamycin treatment.

Our results showed marked decrease in infarct size as a result of Rapamycin treatment at the onset of reperfusion. Furthermore, apoptotic cell death was decreased, indicating an induction in pro-survival protein cascades and/or inhibition of pro-apoptotic pathways. The infarct region is deadly both during the AMI, and after, as it has the potential to drive the heart into pathological remodeling. Improper cardiac hypertrophy or ventricular dilation drastically impede the contractile ability of the heart, often leading to heart failure, and death.[5] However, cardiac remodeling is an important stress recovery mechanism and, while too much can be deadly, too little would result in a much quicker death.

mTOR serves to regulate anabolic and catabolic cellular states, and provide a therapeutic target with which to facilitate proper myocardial recovery. It plays a key role in cardioprotective pressure-induced cardiac hypertrophy, and complete deletion of mTOR

has been proven to cause lethal dilated cardiomyopathy.[19,22,31] mTOR is divided into two distinct complexes: the Rapamycin-sensitive mTORC1 and the Rapamycin-insensitive mTORC2. In the present study, S6 phosphorylation (by mTORC1) exhibited an increasing trend following *in vivo* I/R, suggesting that mTORC1 activation is the primary complex stimulated during I/R injury. Following Rapamycin treatment, the dramatic decrease of pS6 and total S6 further substantiates this. S6 is an upstream inhibitor of autophagy, allowing us to infer that as Rapamycin interferes with mTORC1 and increased autophagy levels may offer some cardioprotective benefit during reperfusion. Akt, the downstream target of mTORC2, promotes cell survival and cytoskeletal growth, as well as restricting Beclin-1 function.[36] Our results showed Akt phosphorylation was significantly increased with Rapamycin. Thus, while mTORC1 inhibition appears to assist with the minimization of reperfusion injury, mTORC2 activation plays a dominant role in I/R cardioprotection.

In addition to the PI3K-Akt signaling pathway, the MAPK family of kinases also regulate cell survival and cell death. JNK and P38 MAPK are the members of the stress-sensitive kinase family that respond to various environmental stimuli such as cytokines, radiation, and reactive oxygen species. The MAPKs regulate the critical cellular processes such as gene transcription, cytoskeletal organization, metabolic homeostasis, cell growth, and apoptosis via a complex signal transduction cascade, which most likely occurs by phosphorylation of some common downstream target proteins. Although it is generally agreed that P38 MAPK plays a pivotal role in the signaling mechanisms of I/R injury, the

question of whether p38 MAPK phosphorylation is protective or detrimental continues to be debatable.

JNK and P38 also act as mediators of P53 activation and cell death in response to stress and DNA damage, noting a marked increase during events such as I/R.[3,4] ERK, also a downstream target of Akt, exhibits both pro-survival and pro-apoptosis characteristics, but recent research has shown significant ERK induction during Rapamycin-facilitated preconditioning.[19] In the present study, total JNK levels were significantly increased with *in vivo* I/R exposure, but were not affected by Rapamycin. Our data showing attenuation of P38 phosphorylation in the Rapamycin treatment group contradicts earlier literature, but provides direct evidence that P38 and, most likely, P53-mediated cell death are inhibited by Rapamycin. Earlier studies showing increased phosphorylation of ERK with Rapamycin were supported by our results, and are indicative of pro-survival STAT3 activation.[54] Additionally, a strong link can be established with mTORC2-induced Akt and downstream ERK phosphorylation. In regards to the MAP kinases, it is apparent that Rapamycin administered during reperfusion activates ERK, concurrently inhibits P38, and has no significant effect on JNK *in vivo*.

The STAT3 protein, a member of the JAK-STAT signal cascade, is localized both the nucleus and mitochondria. It is a downstream activation target of ERK which inhibits MPTP opening and, therefore, apoptosis. In the diabetic model, STAT3 induction increased with Rapamycin treatment, yet the results of this study indicated a different set of changes in healthy mice.[54,56] In these hearts, I/R appears to be the driving force for

STAT3 induction, while the addition of Rapamycin caused no significant changes. Thus, it appears that different metabolic states pose radically different mechanisms of damage repair in I/R injury. Future studies are required to better compare these individual pathways.

It has been shown that STAT3 is responsible for the upregulation of the anti-apoptotic Bcl-2 and pro-apoptotic Bax mitochondrial proteins. Interestingly enough, Bcl-2 levels were not affected by Rapamycin, yet Bax did see a significant reduction in protein expression. While there is only an upwards trending Bcl-2/Bax ratio recovery with Rapamycin, we believe that additional signal pathways, such as the PI3K-Akt pathway, further regulate these mitochondrial proteins.

In conclusion, we have provided several novel insights into the complex mechanisms by which Rapamycin treatment during reperfusion bestows cardioprotective benefits *in vivo*. While mTORC1 is inhibited, mTORC2 activity is amplified. Akt appears to be the mediator between the PI3K-Akt mTOR cascade and the MAP kinases. STAT3 is upregulated by I/R stress alone, and does not appear to play a significant role in Bcl-2/Bax regulation compared to the PI3 kinases. At the present time, further studies are necessary to better understand the effects of metabolic dysregulation on mTOR mediated cardioprotection following reperfusion.

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- **Dean's List** – Spring 2014 – Spring 2015

Peer-Reviewed Publications

Das A, Salloum FN, Filippone SM, et al. Inhibition of mammalian target of rapamycin protects against reperfusion injury in diabetic heart through STAT3 signaling. *Basic Res Cardiol*. 2015;110(3):1-11.