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Divergent Roles of PI3K and Akt in Rapamycin-induced Cardioprotection against Ischemia-Reperfusion Injury

Shivani Kirit Desai
Virginia Commonwealth University

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DIVERGENT ROLES OF PI3K AND AKT IN RAPAMYCIN-INDUCED CARDIOPROTECTION AGAINST ISCHEMIA-REPERFUSION INJURY

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology at Virginia Commonwealth University

by

SHIVANI KIRIT DESAI

The College of William and Mary, B.S. Biology, 2005

Director: Dr. Rakesh C. Kukreja, Ph.D.
School of Medicine
Division of Cardiology

Virginia Commonwealth University
Richmond, Virginia
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4E-BP1	4E binding protein 1
BSA	bovine serum albumin
CF	coronary flow
CHD	coronary heart disease
DF	developed force
DMSO	dimethyl sulfoxide
eNOS	endothelial nitric oxide synthase
GSK3β	glycogen synthase kinase 3
HR	heart rate
i.p.	intraperitoneally
IPC	ischemic preconditioning
I-R injury	ischemia-reperfusion injury
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor-κB
NO	nitric oxide
p70S6K	protein 70S6 kinase
PDK1	phosphoinositide-dependent kinases 1
PI3K	phosphatidylinositol 3-kinase
RAPA	rapamycin
RFP	rate force product
SEM	standard error of mean
TBST	Tris-buffered saline and Tween-20
TGS	Tris/glycine/SDS
TTC	triphenyl tetrazolium chloride
WTN	wortmannin

ABSTRACT

DIVERGENT ROLES OF PI3K AND AKT IN RAPAMYCIN-INDUCED CARDIOPROTECTION AGAINST ISCHEMIA-REPERFUSION INJURY

By Shivani K. Desai, B.S.

A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science in Physiology at Virginia, Virginia Commonwealth University.

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Major Director: Dr. Rakesh C. Kukreja, Ph.D.
VCU School of Medicine
Division of Cardiology

Coronary heart disease (CHD) is one of the leading causes of death every year with nearly three-fourths of all deaths caused by the disease. The challenge scientists are facing today is discovering new drugs to protect the heart against cellular damage caused by ischemia-reperfusion injury (I-R injury). Rapamycin is one such drug that has been shown to protect the heart against ischemia-induced cellular injury. Rapamycin

(sirolimus) inhibits protein synthesis through inhibition of the mammalian target of rapamycin (mTOR). This property of rapamycin has led to its current clinical applications in drug-eluting stents and in immunosuppresive treatment to organ transplant patients. The mechanism by which this drug protects against I-R injury is currently unknown. The goal of this study is to elucidate rapamycin's cardioprotective signaling pathway. We hypothesized that upregulation of Akt occurs possibly as part of a positive feedback mechanism following the inhibition of mTOR by rapamycin. Adult male ICR mice were treated with rapamycin (0.25 mg/kg, *i.p.*), or volume-matched DMSO (solvent for rapamycin), or rapamycin (0.25mg/kg, *i.p.*) plus wortmannin (WTN, 15μg/kg, *i.p.*), an inhibitor of phosphatidylinositol 3-kinase, or wortmannin alone (15μg/kg, *i.p.*). After 30 min of stabilization, the hearts were subjected to 20 minutes of global ischemia and 30 minutes of reperfusion in Langendorff model. In a separate series of experiments mice were either injected with DMSO or rapamycin for 30 minutes, 1 hour, and 2 hours before harvesting the hearts for Western blot analysis of levels of total or phosphorylated Akt at Ser[473]. Our results showed that rapamycin protected the heart as observed by a reduction in infarct size from $33.8 \pm 2.0\%$ in DMSO-treated hearts to $19.3 \pm 4.1\%$ in rapamycin-treated hearts; a 43% reduction. This infarct-limiting effect was completely blocked by wortmannin ($29.3 \pm 4.8\%$). However, Western blot analysis showed no change in the level of Akt phosphorylation after administration of rapamycin. Our current results further confirmed rapamycin as a potential cardio-therapeutic drug to limit infarct size, potentially through the PI3K signaling pathway. However, the exact signaling pathway of this protection still remains elusive.

INTRODUCTION

1.1 Coronary Heart Disease

In 2004, the American Heart Association determined coronary heart disease (CHD), the cause of heart attacks and angina, to be the single leading cause of death in America. About 325,000 people a year die of CHD which is nearly 75% of all deaths. All in all, that is nearly 900 Americans each day, most of whom die from sudden cardiac arrest. Acute coronary occlusion is expected to be the major cause of death by the year 2020 according to the World Health Organization (19; 60). With this meek prediction, new medical therapies are urgently needed to deal with the consequences of CHD in order to reduce the global impact of this disease.

As mentioned, CHD is the cause of heart attacks or myocardial infarctions which occur when there is a sudden occlusion of one or more of the coronary arteries. This blockage reduces or eliminates the flow of oxygenated blood to the myocardium normally supplied by the occluded vessel. Myocardial infarctions are usually caused by a condition called atherosclerosis, a buildup of cholesterol and fibrous tissue in the form of plaques on artery walls. The plaques may rupture the artery, causing a blood clot or thrombus to form and block the artery. The lack of blood flow and oxygen is called ischemia and if maintained for more than a few minutes, an ischemic cascade is triggered in which myocytes suffer permanent damage and die mainly through necrosis. As the division of surrounding myocytes cannot replace these dead myocytes, the

preservation of myocardium's viability after ischemia has become a major target for therapeutic research (60).

1.2 Reperfusion injury

In an effort to find a therapy to limit infarct size, in 1983 Jennings and Reimer demonstrated that reperfusion of the occluded vessels was essential to protecting the ischemic myocardium (21). Thereafter, therapies such as thrombolysis (19), anti-platelet therapy (22) and primary angioplasty (19; 32) were used in the clinical setting to re-establish coronary blood flow. Paradoxically, reperfusion therapy itself results in cardiomyocyte death, an inherent phenomenon termed reperfusion injury.

Reperfusion injury causes rapid disruption of heart tissue as shown by the release of cytosolic enzymes and changes in the microscopic structure of the myocytes. Two forms of cell death have been implicated with reperfusion (17). Studies have identified necrosis as one of these forms of cell death, involving tissue edema, matrix degradation, cell lysis and fragmentation, and inflammation (1; 19; 54). Reperfusion also produces a burst of free radical oxidative species and calcium overload (3), which contributes to necrosis (54). Apoptotic cell death is also either triggered or exacerbated during reperfusion (1). This finding is supported by the fact that apoptosis is an ATP-dependent process and diminished ATP levels due to ischemia are restored during reperfusion (19). Although the restoration of blood flow was an important area of investigation, in order to reduce infarct size, discovering new means of cell protection became one of the focused topics in cardiovascular research.

1.3 Ischemic preconditioning

Knowledge about ischemia-reperfusion injury until the mid-1980s had led scientists to believe that a brief period of ischemia sublethally injured the myocardium and made it more sensitive to subsequent periods of ischemia, culminating in infarction. Then, in 1986, Murry et al. discovered that not all myocardial ischemia is harmful. The phenomenon of ischemic preconditioning (IPC) was found in which brief periods of ischemia actually protect the heart against subsequent prolonged ischemia (32; 36; 59; 60). This intrinsic protection has been found to have a biphasic window: an early phase or classical preconditioning that last up to ~2 hours after the preconditioning stimulus followed a day later by a delayed phase or second window of protection (SWOP) lasting ~3days (34; 42; 57; 60).

By effectively reducing both necrosis (32; 59) and apoptosis within an infarcted heart (57), IPC increases the time available for effective reperfusion. Patients with unstable angina and those undergoing bypass surgery or coronary angioplasty can benefit from preconditioning (32). Furthermore, this phenomenon has been shown to occur in all species tested including man and with a very similar reduction in infarct size in all species (42). For these reasons, IPC has been recognized as "the strongest form of in vivo protection against myocardial ischemic injury other than early reperfusion" (28).

Remarkably, the cardioprotective effects of IPC can be induced through the administration of exogenous pharmacological agents such as bradykinin (32; 42), adenosine (42), opioids (42), erythropoietin (15), acetylcholine (6; 42), and nitric oxide (NO) (31). Mitochondrial K_{ATP} channel openers (1; 39; 58), HMG-CoA reductase inhibitors such as simvastatin (23) and atorvastatin (11), phosphodiesterase 5 inhibitors

such as sildenafil (40), and certain volatile anesthetics including desflurane (52) and isoflurane (29) can also mimic IPC's effects. These mimetics have been discovered over the past two decades through investigation of the diverse signaling pathways involved in the biochemical mechanism of IPC and allow us to prolong the short-lived effects of acute IPC (57).

1.4 Phosphatidylinositol 3-kinase/Akt signaling pathway

Tong et al. were the first to implicate the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway during IPC (55; 56). PI3K, a lipid kinase (2), has a vital role in mediating many cellular responses in both physiologic and pathophysiologic states such as cell cycle progression, differentiation, and survival (14). Stimulation of cell surface receptors activates PI3K, which then itself phosphorylates membrane phophoinositides at the D-3 position. These phospholipids act as second messengers that mediate the diverse cellular functions of PI3K (2; 24). One function of these second messengers is the activation of the serine/threonine kinase Akt, another name for which is protein kinase B (7). Akt contains a pleckstrin homology domain on its amino terminus that directly binds the phospholipid products of PI3K. As a result, Akt is recruited from its cytoplasmic location to the membrane where these PI3K products are located (53). This allows a conformational change in the PI3K products, enabling the phosphorylation of Akt by the phosphoinositide-dependent kinases 1 (PDK1) and PDK2 at residues Thr[308] and Ser[473], respectively. Full activation of Akt is due to the phosphorylation of these two residues (2; 14; 24; 27). In turn, activated Akt phosphorylates a number of downstream targets.

1.5 Akt signaling effects

Akt is a multifunctional protein kinase with diverse downstream target effects that can be roughly divided into three groups: 1) anti-apoptotis/cell survival, 2) cellular growth and metabolism, and 3) increased translation. The cardioprotective effects of Akt activation have been mainly attributed to the reduction of myocardial apoptosis. Akt's anti-apoptotic actions involve both cytoplasmic and nuclear compartments (7). Akt phosphorylates the protein BAD at Ser^{136} (22) which inhibits BAD's pro-apoptotic activity by reducing its inhibition of the anti-apoptotic proteins, Bcl-2 and Bcl-X_L (41; 53; 57). Other targets include the forkhead transcription factors: FKHR, FKHR-L1, and AFX, which are all, down-regulated when Akt phosphorylates them (2; 41). For example, phosphorylation of FKR-L1 retains it in the cytoplasm through sequestration by 14-3-3 proteins and prevents it from activating transcription of pro-apoptotic genes (53).

Akt also promotes cell survival by maintaining the integrity of the outer mitochondrial membrane. By doing so, cytochrome *c* release into the cytosol is prevented. Normally if cytochrome *c* were allowed to be released into the cytosol, it would bind to and activate the apoptotic protease-activating factor (Apaf-1), which in turn cleaves and activates caspase-9, resulting in the activation of caspase-3 and caspase-7. At this point the cells are committed to apoptosis (24; 43; 57). As for Akt target effectors in the nucleus, Akt activates IκB kinases that degrade the inhibitory protein IκB. IκB keeps the transcription factor, nuclear factor-κB (NF-κB), in the cytoplasm. Once released, NFκB is free to migrate to the nucleus and regulate transcriptional activity of inhibitor-of-apoptosis proteins (IAPs) (41; 53).

Akt has many other cardiovascular functions by activating proteins that regulate cellular proliferation, migration, and growth (41; 49). Glycogen synthase kinase 3 (GSK3β) is a growth inhibitory protein (2) and well-defined direct target of Akt. When Akt phosphorylates GSK3β, it reduces its kinase activity (49), thus promoting glycogen synthesis (50). Past studies suggest that an increase in cardiac glycogen might increase resistance to cellular hypoxia during ischemia by providing a greater pool of fuel reserve for anaerobic glycolysis (53).

Akt also has a key role in vascular homeostasis and angiogenesis (1; 50; 57). Several stimuli in endothelial cells activate the PI3K/Akt signaling axis and regulate endothelial cell survival, migration, and capillary-like structure formation, all important steps in angiogenesis. Additionally, Akt controls NO synthesis by phosphorylating endothelial nitric oxide synthase (eNOS), which has been associated with delayed preconditioning (26). An increase in NO synthesis results in regulation of cardiovascular homeostasis, vessel integrity, and vasomotor tone by increasing resting blood vessel diameter and blood flow (50). Finally, a recent study demonstrated a marked increase in heart size in mice expressing constitutively active Akt (49). This finding exemplifies Akt's promotion of organ growth in vertebrates. Moreover, Akt's role in angiogenesis implies that it must regulate this process during organ enlargement (50).

The cardioprotective effect of Akt activation after transient ischemia (33) may render it a critical control point determining not only cardiomyocyte survival but, function as well. Oudit et al. found constitutively active Akt genes to be influential on myocardial contractile force although the effects were variable (41). This ultimately makes Akt an important target for therapy in ischemic heart disease. The time dependent

activation of Akt by IPC mimetics has been researched in the past. Ahmad et al. (1) and Wang et al. (58) tested the time-dependency of Akt phosphorylation by BMS-191095 and diazoxide, respectively. BMS-191095 and diazoxide are two mitoK$_{ATP}$ channel openers known to protect the heart against I-R injury. It has already been shown that a variety of agents mimicking IPC are able to minimize I-R injury by the activation of prosurvival kinases. Yellon et al. demonstrated the cardioprotective effect of atorvastatin given at the commencement of reperfusion in the intact mouse heart and associated this protection with the upregulation of PI3K/Akt (11).

1.6 Mammalian target of rapamycin

A final Akt downstream target, the mammalian target of rapamycin (mTOR), is a 289-kDa serine/threonine kinase. Akt activation of mTOR via phosphorylation (38) leads to an altered metabolism and increased growth mediated by changes in gene transcription and translation. Activated mTOR can phosphorylate and thereby inactivate the eukaryotic translation initiation factor called 4E-binding protein 1 (4E-BP1), resulting in increased protein translation (2; 3; 50). mTOR also phosphorylates and activates ribosomal protein 70S6 kinase (p70S6K) at Thr[389] (14; 18). Activated p70S6K phosphorylates ribosomal protein S6 which increases the translation of 5'–terminal oligopyrimidine (TOP) tract mRNAs, thus enhancing the synthesis of translational machinery (2; 49; 50). Like Akt, p70S6K also inactivates the pro-apoptotic peptide, BAD (16), by phosphorylating its Ser[136] residue (22). These effects are crucial to cellular growth and enhancing protein synthesis. However, the present study centers on the inhibition of mTOR by the drug, rapamycin.

1.7 Rapamycin

Rapamycin is a macrolide ester (38) produced by a strain of *Streptomyces hygroscopicus* which was isolated in 1975 from a soil sample collected from Rapa Nui, better know as Easter Island. These days, the natural product has the generic name sirolimus and has acquired the trademark name Rapamune® (47; 48). It was first isolated as an antifungal agent however, additional studies have shown the drug to have anti-tumor and immunosuppressive effects (3; 10). Rapamycin's clinical relevance includes its ability to prevent restenosis, a prevalent side-effect after angioplasty surgery that occurs in 30% to 60% of patients within the first six months. Because the drug is released locally, sirolimus-coated stents effectively reduce the rate of restenosis (41; 51). The antibiotic's unique mechanism of action has caused it to pass clinical trials and is today approved as an immunosuppressive therapy in organ transplantation (47; 48).

Rapamycin is known to bind with high affinity to its intracellular receptor, the immunophilin (2) FK506-binding protein FKBP12. This complex binds to mTOR, inhibiting its function and allowing dephosphorylation of p70S6K and 4E-BP1 (3; 20). Literature has shown results which suggest that inactivating mTOR/p70S6K may mediate the beneficial effects of preconditioning and several preconditioning mimetic drugs. One study found that the inhibition of p70S6K prevented the phosphorylation of BAD and blocked cell survival induced by insulin-like growth factor 1(IGF-1) (16). Another study demonstrated how prolonged activation of p70S6K can cause cardiac hypertrophy (46), suggesting that inhibiting the mTOR/p70S6K complex with rapamycin may have beneficial cardiovascular effects (13; 26).

Our lab (Khan et al.) was the first group to demonstrate the cardioprotective effect of rapamycin against I-R injury in the isolated mouse heart and in cardiac myocytes subjected to hypoxia-reoxygenation injury (13; 25). A significant reduction in infarct size was observed but, there was no improvement in heart function. In support of this result, another group discovered rapamycin's ability to inhibit apoptosis by inhibiting phosphorylation of p53 which blocks the transcriptional activation of pro-apoptotic proteins such as Bax and the activation of the mitochondrial cell death process (5).

The drug's effect seems to be dose-dependent as lower doses (1 nM) blocked protection. In this study we used a previously reported cardioprotective dose (0.25mg/kg) (25). In most studies that show rapamycin to block cardioprotection, the drug is administered at or before reperfusion. In our previous study, rapamycin was administered as a pretreatment (25). All in all, drugs that inhibit mTOR such as rapamycin may have either beneficial or harmful effects depending on the dose and timing of administration (13).

1.8 Present study

The aim of this study is to understand the role of PI3K/Akt phosphorylation in the cardioprotection of rapamycin and better understand why the drug has varying effects. We hypothesize that rapamycin could possibly influence the cross-talk between the survival kinases whereby inhibition of mTOR may result in the upregulation of other prosurvial knases such as Akt. Until recently, it was thought that Akt directly phosphorylated its downstream target mTOR (38). Interestingly, research has proved Akt to remain active following treatment of rapamycin (20). This could indicate one of two

things: either a possible Akt-independent mechanism for mTOR phosphorylation or a possible positive feedback loop from rapamycin-inactivated mTOR to Akt. A study in 2005 demonstrated that blocking the PI3K/Akt/mTOR pathway results in the partial reduction of the development of cardiac hypertrophy in vivo (14). In order to test the role of PI3K, we used wortmannin (WTN). WTN inactivates PI3Ks by covalent modification of the catalytic subunit p110 (41). We used our well-established Langendorff isolated perfused mouse heart model of global ischemia-reperfusion, which has been used extensively to probe the therapeutic efficacy of drugs.

Figure 1: PI3K/Akt signaling pathway
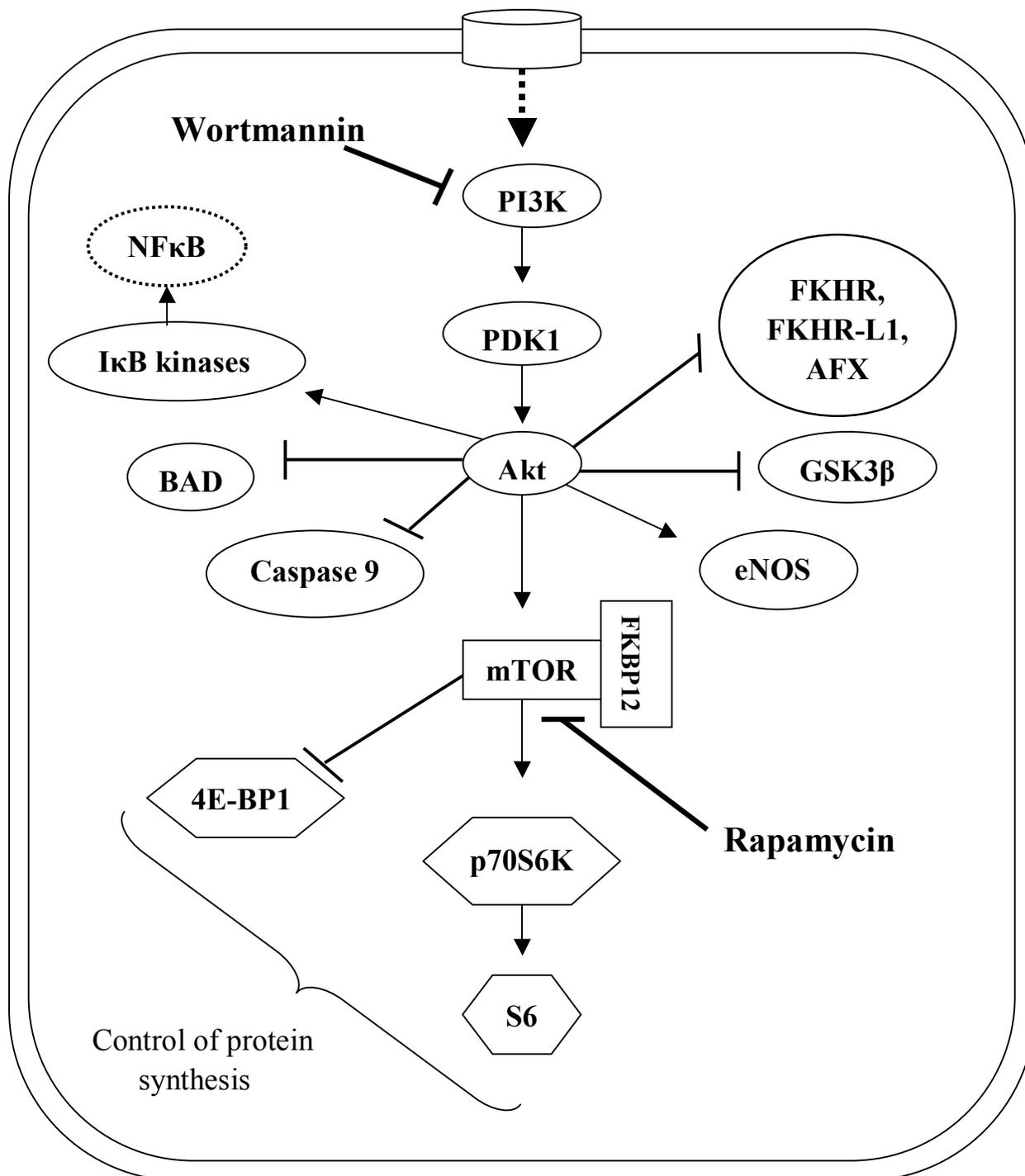
MATERIALS AND METHODS

2.1 Animals

Adult male ICR mice weighing 35.5 ± 1.2 g were supplied by Harlan Sprague Dawley Co. (Indianapolis, IN). All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2 Drugs and chemicals

Rapamycin and wortmannin were purchased from Sigma-Aldrich (St. Louis, MO) and were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) for intraperitoneal injection (*i.p.*). Final concentrations for rapamycin and wortmannin were 5.55% and 0.25%, respectively.

2.3 Langendorff isolated heart preparation

The methods for the isolated, perfused mouse heart preparation were previously described in detail (25; 59). Prior to isolating the heart, the animal was weighed and anesthetized. Anesthesia was prepared by mixing 0.2cc of heparin to protect the heart

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against microthrombi, 0.4cc of pentobarbital, and 0.2cc saline for 4 mice. Each mouse was injected intraperitoneally with 0.2cc of anesthesia, the equivalent of 100mg/kg of sodium pentobarbital per mouse. The chest was then opened at the sternum, and the heart was rapidly removed from the thorax and placed in a small dish containing ice-cold Krebs-Henseleit (K-H) buffer and heparin (0.1cc per dish), where it was trimmed of excessive tissue and fat. Using an illuminated magnifier (4X), the aortic opening was exposed and quickly cannulated on a 20-gauge blunt needle and double loop tied using no. 5 surgical thread.

The heart was then retrogradedly perfused at a constant pressure of 55 mmHg through the aorta in a non-recirculating Langendorff apparatus (Figure 2) with modified K-H buffer containing (in mM) 118 NaCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 4.7 KCL, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, and 0.5 EDTA. The K-H solution was pre-filtered by a micro-filter (Millipore Corp.) with a pore size of 0.45µm diameter, and was continuously gassed with 95% O_2 + 5% CO_2 (pH ~ 7.4). The perfusion solution was warmed to 40ºC through a water-jacketed glass cylinder/heat exchanger system with a warming/cooling bath and automatic thermal controller (Brinkmann) and was constantly circulated by a varistaltic water pump (Manostat). The solution was warmed to 40ºC to compensate for any heat loss experienced as the buffer circulates through the Langendorff apparatus. The ambient temperature around the heart was also kept at 37 ± 0.2 ºC throughout the experiment using a 100 Watt heating lamp. A digital thermometer (VWR) continuously monitored the temperature around the heart. During the no-flow ischemia, K-H buffer was periodically applied on the heart surface to keep it moist. Hearts demonstrating persistent arrhythmia during the pre-ischemia period were excluded from further study.

Figure 2: Langendorff isolated perfused heart system
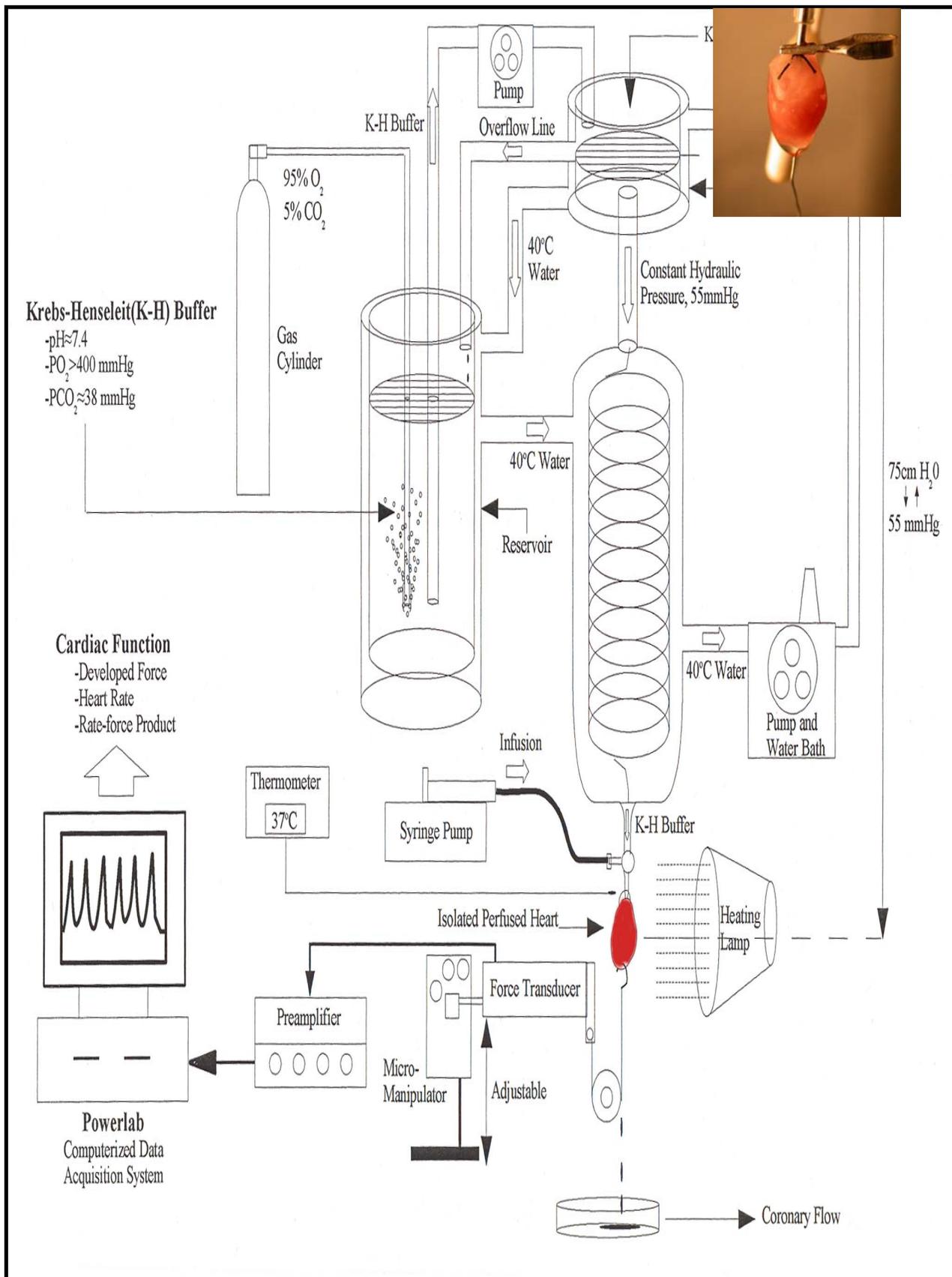
2.4 Assessment of heart function

Following the start of perfusion, ventricular function was measured by a force-displacement transducer (Grass, Model FT03) attached to the apex of the mouse heart through a rigid metal hook attached to a no. 5 surgical thread. The resting tension of the isolated heart was adjusted to 0.3g. Ventricular developed force was continuously recorded with a PowerLab 8SP computerized data acquisition system (AD instruments) connected to the force transducer. The coronary flow was calculated by timed collection of the efflux perfusate at 15 minutes of stabilization (for pre-ischemia value) and again at 15 minutes of reperfusion (for post-ischemia value). The hearts were not electronically paced.

2.5 Experimental protocol for drug pretreatment and cardiac ischemia-reperfusion

To investigate whether the cardioprotective effect of rapamycin is mediated by the PI3K/Akt signaling pathway, we administered wortmannin, a PI3K inhibitor, in addition to rapamycin and the vehicle DMSO, intraperitoneally. As diagrammed in Figure 3, a total of 29 mice were randomly assigned to four groups. In Group 1 (n=6), DMSO was administered at two different time points at two different doses. The first dose was equivalent to the DMSO dilution volume for dissolving WTN (0.25% DMSO in saline, DMSO-W). The second dose was given an hour later and was equivalent to the DMSO dilution volume for dissolving rapamycin (5.55% DMSO in saline, DMSO-R). In Group 2 (n=8) mice received the DMSO-W dose and thirty minutes later received rapamycin (0.25 mg/kg). Group 3 (n=7) mice were injected with WTN (15µg/kg) and thirty minutes later were injected with rapamycin (0.25 mg/kg). Group 4 (n=7) was

treated with WTN (15µg/kg) alone. All hearts were excised and isolated 90 minutes after the first drug was injected. The hearts were stabilized for 30 minutes after isolation and subjected to zero-flow ischemia for 20 minutes followed by 30 minutes of reperfusion on the Langendorff apparatus.

Table 1:   Practical notes for drug preparation

GROUP 1 DMSO CONTROL	**DMSO-R**: 50µL DMSO plus 850µL saline=0.9mL. Inject 0.15mL of this solution into each mouse (5.55% [DMSO]). **DMSO-W**: 2.5µL DMSO plus 997.5 µL saline = 1mL. Inject 0.2mL of this solution into each mouse (0.25 % [DMSO]).
GROUP 2 DMSO-W + RAPA	**DMSO-W**: 2.5µL DMSO plus 997.5 µL saline = 1mL. Inject 0.2mL of this solution into each mouse (0.25 % [DMSO]). **RAPA**: 1mL DMSO was used to dissolve 1mg vial of rapamycin. 50µL of this solution plus 850 µL saline =0.9mL. 0.15mL of this solution was injected into each mouse (5.55% [rapamycin]).
GROUP 3 WTN + RAPA	**WTN**: 1mL DMSO was used to dissolve 1mg vial of wortmannin. 2.5µL of this solution plus 997.5 µL saline = 1mL. Inject 0.2 mL of this solution into each mouse (0.25 % [WTN]). **RAPA**: 1 mL DMSO was used to dissolve 1mg vial of rapamycin. 50µL of this solution plus 850 µL saline =0.9 mL. 0.15 mL of this solution was injected into each mouse (5.55% [rapamycin]).
GROUP 4 WTN	**WTN**: 1mL DMSO was used to dissolve 1mg vial of wortmannin. 2.5µL of this solution plus 997.5 µL saline = 1mL. Inject 0.2 mL of this solution into each mouse (0.25 % [WTN]).

Figure 3:   Experiment Protocol 1

GROUP 1 DMSO Control

| 1 hour | 30 min | 30 min | Stabilization | Global ischemia 20 min | Reperfusion 30 min |

DMSO-W, i.p. (0.25% DMSO)   DMSO-R, i.p. (5.5% DMSO)   isolate heart   infarct measurement

GROUP 2 DMSO-W + Rapa

| 1 hour | 30 min | 30 min | Stabilization | Global ischemia 20 min | Reperfusion 30 min |

DMSO-W, i.p. (0.25%)   Rapamycin, i.p. (0.25 mg/kg)   isolate heart   infarct measurement

GROUP 3 WTN + Rapa

| 1 hour | 30 min | 30 min | Stabilization | Global ischemia 20 min | Reperfusion 30 min |

WTN, i.p. (15 µg/kg)   Rapamycin, i.p. (0.25 mg/kg)   isolate heart   infarct measurement

GROUP 4 WTN only

| 1 hour 30 min | 30 min | Stabilization | Global ischemia 20 min | Reperfusion 30 min |

WTN, i.p. (15 µg/kg)   isolate heart   infarct measuremen

2.6 Measurement of infarct size

At the end of each experiment, the hearts were immediately removed from the Langendorff apparatus, dried with a Kimwipe, weighed, and frozen at -20°C. The next day, the frozen hearts were cut by hand using a surgical blade parallel to the atrioventricular groove, into 6 to 8 transverse slices of approximately equal thickness (~1mm). The slices were placed onto an inverted petri dish (Becton Dickinson Labware) and put face down into the inverted cover of the same petri dish. The heart slices were stained by incubating them in 10% 2, 3, 5-triphenyl tetrazolium chloride (TTC, Sigma) solution for 30 minutes at room temperature (~22°C).

In normal myocardium, TTC is converted by dehydrogenase enzyme to a dark red formazan pigment. On the other hand, infracted myocardium, due to loss of dehydrogenase enzyme following cell membrane ruptures, does not take up TTC stain and remains a pale color (58). After staining, the TTC was removed and replaced with 10% formaldehyde for additional 2-4 hours to fix the heart slices. Thereafter, the heart slices were digitally imaged and total area, cavities, and infracted area were measured using computer morphometry (Bioquant98) to determine risk area and infarct size. The risk area was calculated as total ventricular area minus the area of the cavities. The infarct size was calculated as a percentage of the risk area.

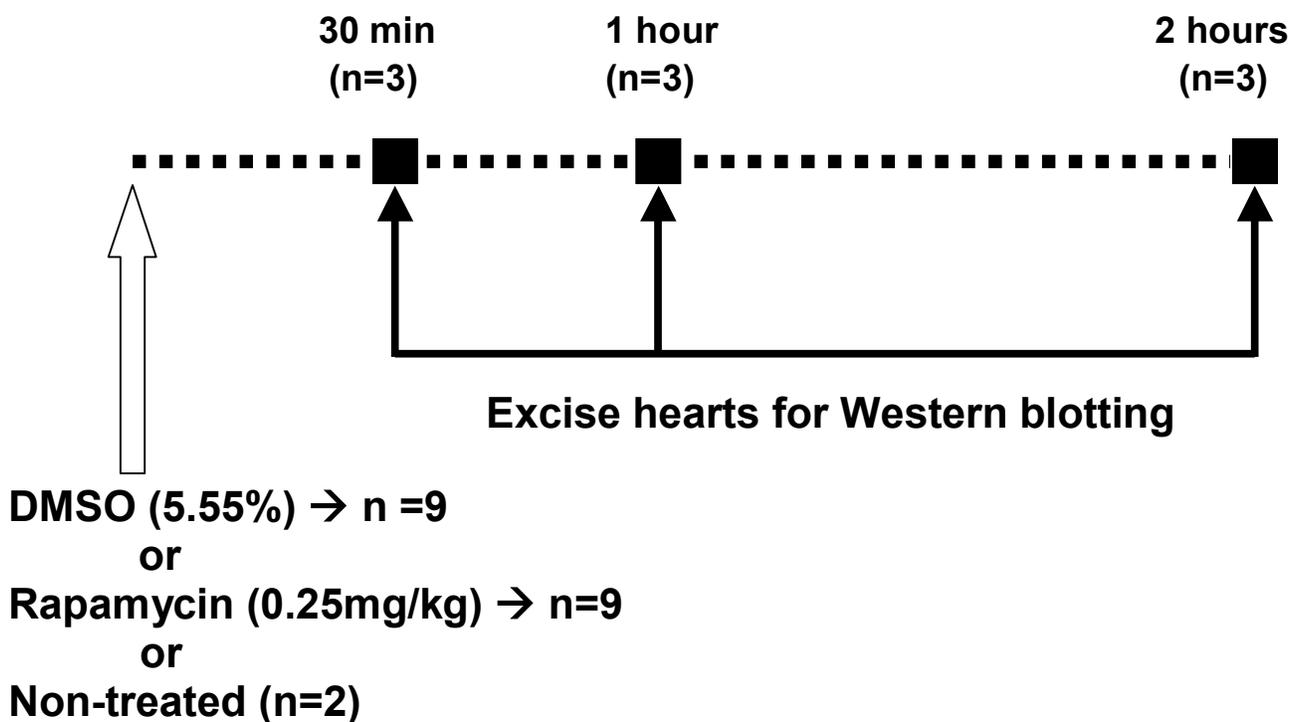*2.7 Protein extraction for Western blot analysis*

A separate subset of mouse hearts was used to determine Akt phosphorylation by rapamycin. Twenty-four (24) mice were anesthetized with sodium pentobarbital (100mg/kg, *i.p.*) and hearts were harvested from the control, DMSO-treated or

rapamycin-treated groups at the different time points shown in Figure 4. Two non-treated mice were included as control samples. The hearts were then snap frozen in liquid nitrogen and immediately stored in −80°C conditions until use. Each heart sample was crushed using a mortar and pestle. Liquid nitrogen was added as needed during this process to prevent the tissue sample from defreezing. Once the sample was crushed into a fine powder, 1.2 mL of ice-cold lysis buffer (Pierce) [containing (in mM) 25 Tris* HCL pH 7.6, 150 NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS] was added to each sample tube and kept on ice for 30 minutes. Halt^{TM} Protease Inhibitor Cocktail Kit (Pierce) and Halt^{TM} Phosphatase Inhibitor Cocktail (Pierce) were also added to the buffer to protect the sample protein from degradation by endogenous proteases, serine/threonine phosphatases and tyrosine phosphatases (10 µL of each inhibitor per mL of RIPA buffer used).

The samples were then homogenized for 3 bursts of 3 to 4 seconds with a Polytron homogenizer and kept on ice for an additional 15 minutes before being transferred to Eppendorff tubes and centrifuged at 14,000 rpm (20800g, Eppendorf Centrifuge 5810R) at 4°C for 10 minutes. The supernatant was separated from the pellet, divided up into 3 aliquots, and stored in −80°C conditions for further analysis. Protein content of each heart sample was determined by using 10 mg of bovine serum albumin (BSA, Sigma-Aldrich) in 10mL of distilled water, forming a BSA stock solution. Taking 1 mL of the solution and adding it to 19 mL of distilled water diluted this stock solution. This second solution was used to establish a standard curve by adding different amounts of the BSA solution, water and protein assay dye (Biorad). To determine the protein concentration of our collected samples, 2 µl of each heart tissue sample were added to

798 µl of water along with 200 µl of protein assay dye before protein concentration was determined by performing a Bradford assay using a SmartSpec^{TM} 3000 spectrophotometer (Biorad).

Figure 4: Experimental Protocol 2

30 min
(n=3)

1 hour
(n=3)

2 hours
(n=3)

Excise hearts for Western blotting

DMSO (5.55%) → n =9
or
Rapamycin (0.25mg/kg) → n=9
or
Non-treated (n=2)

2.8 Western blot analysis for Akt

Western blot analysis was performed to determine the effect of rapamycin and its vehicle, DMSO at different post-treatment time points on total Akt and phosphorylated Akt. Protein samples were combined with an equal volume of a loading buffer solution made by mixing 25 µl of Laemmli sample buffer (Biorad) and 475 µl 2-mercaptoethanol electrophoresis purity agent (Biorad). The samples were boiled for 7-10 minutes and centrifuged for 20 seconds at 14,000 rpm at room temperature before being loaded into each well on 10% polyacrylamide gels (Biorad). A protein marker (Biorad) was also used for better identifying the molecular weight of the target protein. The gels were electrophoresed by a Biorad energy pack for 1 hour at 180 volts using a running buffer [1X Tris/glycine/SDS (TGS)]. These electrophoresed proteins were transferred from the gel to nitrocellulose membranes (Biorad) for 90 minutes at 400 mA in 4°C using a transfer buffer (700 mL water, 200 mL methanol, 100 ml 10X TGS). After transfer, the nitrocellulose membranes were washed for 5 minutes using 1X Tris-buffered saline (Biorad) and 5% Tween-20 (Biorad) wash (TBST). The membranes were incubated for 1 hour in 20 mL of a blocking solution [5% blotting milk (Biorad), 20 mL of TBST] at room temperature to block nonspecific binding sites. Subsequently, the membranes were incubated with a goat polyclonal antibody (Santa Cruz) specific for total Akt or a rabbit polyclonal antibody (Santa Cruz) specific for phosphorylated Akt each in 1:1000 dilutions (i.e. 20 µl antibody in 20 mL of blocking solution) on a rocking platform overnight in a 4°C cold room. The housekeeping gene, β-actin (Santa Cruz), was also blotted to demonstrate the protein loading condition in each gel lane.

The next day, the blots were washed thoroughly with approximately 15 mL of TBST three times for 7-10 minutes each. The blots were then incubated in 1:1000 dilutions of horseradish-labeled anti-goat secondary antibody (Santa Cruz) and anti-rabbit secondary antibody (Amersham) in 20 mL of blocking buffer for 2 hours at room temperature. After a thorough wash with TBST again three times for 7-10 minutes each, the blots were exposed to enhanced chemiluminescence (ECL, Amersham) for a few seconds, wrapped in plastic wrap and taped down in a developing cassette. In a dark room, each membrane was exposed to film for approximately one minute for total Akt and phosphorylated Akt and 20-30 minutes for β-actin before being developed by a film processor (Kodak). The bands of proteins were visualized and quantified using the Image J (Image Processing and Analysis in Java) computer program.

2.9 Data analysis and statistics

The difference between groups was analyzed by unpaired t-tests. $P < 0.05$ was considered to be statistically significant. All values are expressed as means ± SEM.

RESULTS

3.1 Exclusions

A total of 33 mice were used in the isolated perfused heart study and were used for data analysis. Among these, 4 hearts (12% of the 33 perfused hearts) were excluded from various experimental groups due to one or more of the following reasons: damage of the aorta during cannulation, excessive coronary flow (>6 mL/min), longer cannulation time (>3 min), arrhythmic heart beat during the equilibration period. Twenty-four mice were used for Western blot analysis and no mice were excluded from this experiment.

Table 2: Morphometric characters of mice used in isolated heart experiment

Experimental Groups	Body weight (g)	Heart Wet Weight (mg)
DMSO (n=6)	33.5 ± 3.5	237 ± 15
RAPA (n=8)	38.6 ± 3.5	230 ± 11
WTN+RAPA (n=7)	34.7 ± 0.8	203 ± 6
WTN (n=7)	36.8 ± 0.6	221 ± 9

Values shown as means $\pm$ standard error of mean (SEM).

*3.2 Rapamycin preconditioning reduced infarct size*

Previously, rapamycin has been shown to have a cardioprotective effect in the isolated mouse heart model. This was demonstrated by a reduction in infarct size in rapamycin-treated mice (25). As shown in Figure 5A, the infarct size in DMSO control mice was $33.8 \pm 2.0\%$ of risk area. As targeted, rapamycin significantly (*P*=0.0149) decreased infarct size to $19.3 \pm 4.1\%$, a 43% reduction compared to the DMSO control group. Infarct size was calculated as a percentage of total risk area, a measured area that did not vary among the four experimental groups (Figure 5B). This degree of protection by rapamycin is comparable to the highly selective mitochondrial K_{ATP} channel opener, diazoxide (54% decrease). Like rapamycin, diazoxide reduced infarct size from $27.8 \pm 4.2\%$ to $12.9 \pm 1.2\%$ after pharmacological preconditioning with diazoxide (39).

3.3 Wortmannin blocked rapamycin protection

Wortmannin has been used effectively in past studies as a specific PI3K inhibitor (44), blocking signaling to all downstream effectors including Akt (11). To verify the potential Akt mediation of rapamycin protection, wortmannin was used to block PI3K and therefore all proteins downstream including Akt. Figure 5A illustrates the similarity in infarct size between the DMSO control and Group 3 mice which were treated with wortmannin and rapamycin ($29.3 \pm 4.8\%$). As predicted, wortmannin effectively abrogated rapamycin-mediated cardioprotection.

3.4 Wortmannin alone protected the heart

Interestingly, there was a significant (P=0.0403) reduction in infarct size (24.7 ± 3.2%) in the group treated solely with wortmannin (Figure 5A). This result suggests that wortmannin may have cardioprotective capabilities against I-R injury.

A

B
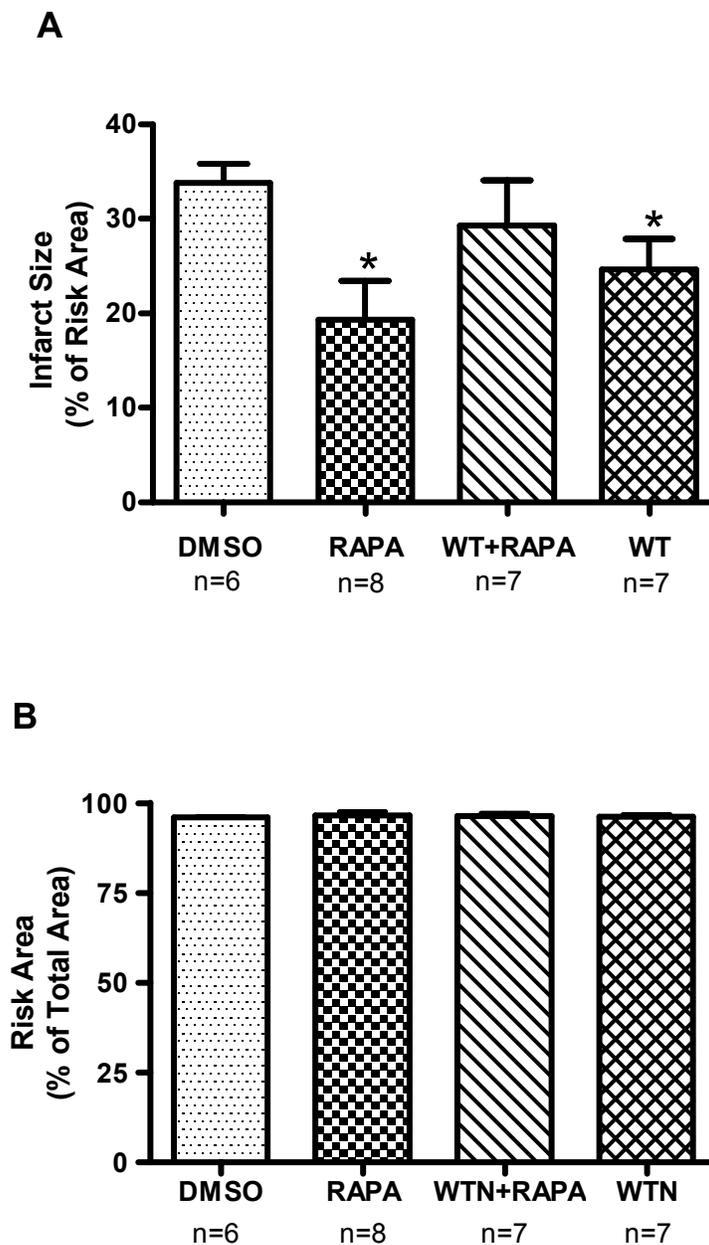
Figure 5A: Myocardial infarct size

Mice were divided into 4 groups and hearts were isolated and perfused as described in chapters 2.3 and 2.5. Infarct size was significantly reduced in two different groups, RAPA-treated and WTN-treated mice (*$P<0.05$ vs. DMSO). Wortmannin also proved to effectively block rapamycin protection.

Figure 5B: Risk area

Risk area was calculated as a percentage of total area and was similar in all four experimental groups.
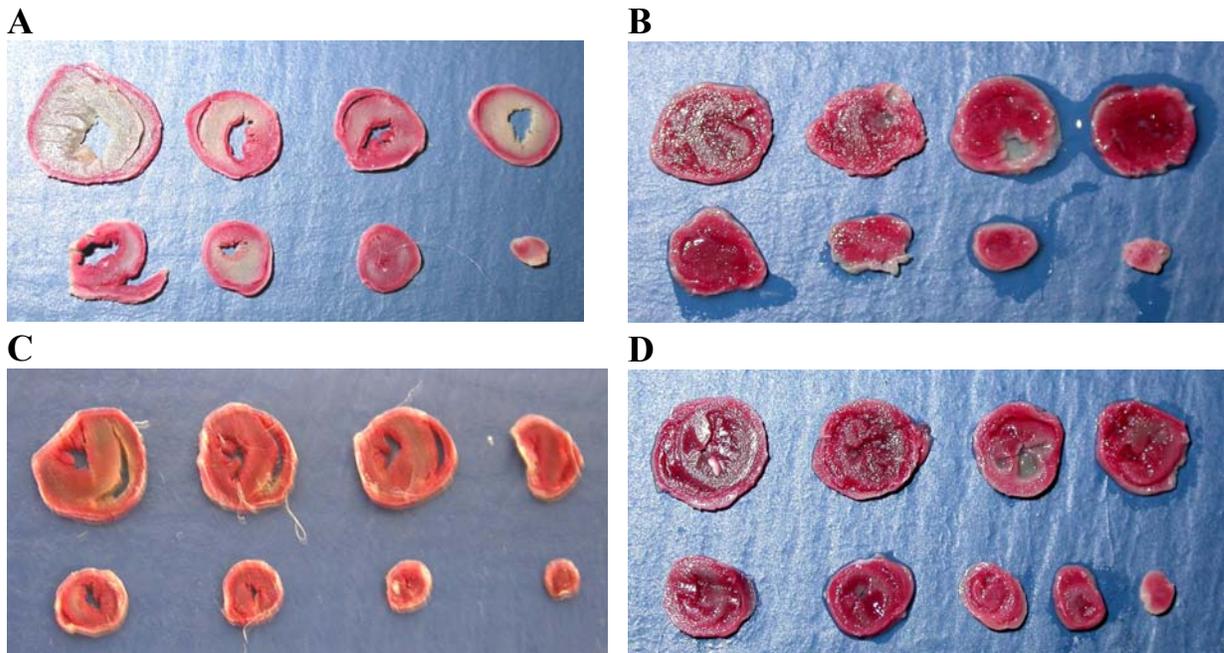
Figure 6: Representative digital images of stained heart slices
A) DMSO-treated heart (n=6)
B) RAPA-treated heart (n=8)
C) WTN- and RAPA-treated heart (n=7)
D) WTN-treated heart (n=7)

Heart slices were stained with TTC and fixed with formalin. Viable tissue remains red, whereas infarcted tissue appears pale and colorless. The DMSO control heart (A) has much larger infarcted area as compared to the RAPA-treated heart (B), showing healthy myocardium and little infarcted tissue. The same can be said of WTN-treated hearts (D) which show very little pale tissue. On the other hand, the group treated with WTN and RAPA (C) has a measurable amount of dead tissue which suggests that WTN successfully blocked rapamycin-induced protection.

3.5 Cardiovascular function

Pre-ischemic and post-reperfusion values of hemodynamic and cardiovascular function of the isolated perfused hearts are summarized in Table 2. In order to study the effect of rapamycin on heart function as well as infarct size we used the isolated heart model, eliminating the influence of circulating factors on the heart. Rate-force product (RFP) is a measured estimation of cardiac function and is calculated as developed force x heart rate (g x beats/minute). When RFP was calculated as a percentage of baseline pre-ischemia values, there was no significant change between all four experimental groups (Figure 7). However, observation of the individual RFP values in Table 2 shows a significant decrease in RAPA-treated groups post-reperfusion compared to DMSO-treated groups post-reperfusion (P=0.0099).

Figure 8 shows a significant decrease in developed force (DF) post-reperfusion, between mice treated with DMSO and those treated with RAPA (P= 0.0268). DF is the amplitude of heart contractility independent of heart rate. A constant trend of reduced DF after ischemia can be observed in all the experimental groups.

Coronary effluent was measured for one minute after 15 minutes of stabilization and again 15 minutes after reperfusion as an estimation of coronary flow *in vivo*. There was no significant change in coronary flow from the pre-ischemic baseline measurement to the post-reperfusion measurement in RAPA-treated mice due to variability within this group (Figure 9B). Nevertheless, there is a noticeable increase in coronary flow in the RAPA-treated group compared to the DMSO-treated group during both pre-ischemia and

post-reperfusion (Figure 9A). An unexpected result, illustrated in Figure 9A, is a significant elevation of coronary flow in WTN-treated groups, pre-ischemia ($P= 0.05$) and post-reperfusion ($P= 0.0414$) compared to the DMSO control group. This result adds to and explains the increased RFP in WTN-treated groups.

Table 2: Values of hemodynamic and contractile parameters of isolated perfused hearts after 15 minutes of stabilization (pre-ischemia) and after 30 minutes of reperfusion

	Pre-ischemia				At 30 minutes of reperfusion			
	DMSO	RAPA	WTN+RAPA	WTN	DMSO	RAPA	WTN+RAPA	WTN
HR (beats/min)	321 ± 29	350 ± 22	359 ± 32	350 ± 16	350 ± 27	325 ± 36	396 ± 28	358 ± 12
DF (g)	0.82 ± 0.14	0.61 ± 0.12	0.56 ± 0.20	0.76 ± 0.16	0.67 ± 0.10	0.36 ± 0.07[a]	0.50 ± 0.20	0.55 ± 0.16
RFP(g beats/min)	278 ± 67	214 ± 44	171 ± 48	258 ± 48	231 ± 31	108 ± 22[b]	178 ± 67	189 ± 49
CF (mL/min)	1.38 ± 0.13	2.39 ± 0.52	1.86 ± 0.46	1.81 ± 0.15[c]	1.29 ± 0.09	3.02 ± 0.89	1.71 ± 0.45	1.83 ± 0.20[d]

[a] $P < 0.05$ vs. DMSO post-reperfusion (unpaired *t* test)
[b] $P < 0.05$ vs. DMSO post-reperfusion (unpaired *t* test)
[c] $P < 0.05$ vs. DMSO pre-ischemia (unpaired *t* test)
[d] $P < 0.05$ vs. DMSO post-reperfusion (unpaired *t* test)

HR, heart rate; DF, developed force; RFP, rate-force product; CF, coronary flow. Values shown are means ± SEM. Experimental groups: 1) DMSO = mice treated with vehicle control (0.25% DMSO, *i.p.*) 90 min and 30 min (5.5% DMSO, *i.p.*) prior to ischemia-reperfusion (I-R); 2) RAPA = mice treated with vehicle 90 min (0.25% DMSO, *i.p.*) before I-R and with rapamycin (RAPA, 0.25mg/kg, *i.p.*) 30 min prior to I-R; 3) WTN + RAPA = mice treated with wortmannin (WTN, 15µg/kg, *i.p.*) 90 min prior to IR and with RAPA (0.25mg/kg, *i.p.*) 30 min prior to IR; 4) WTN = mice treated with WTN (15µg/kg, *i.p*) 90 min prior to I-R.
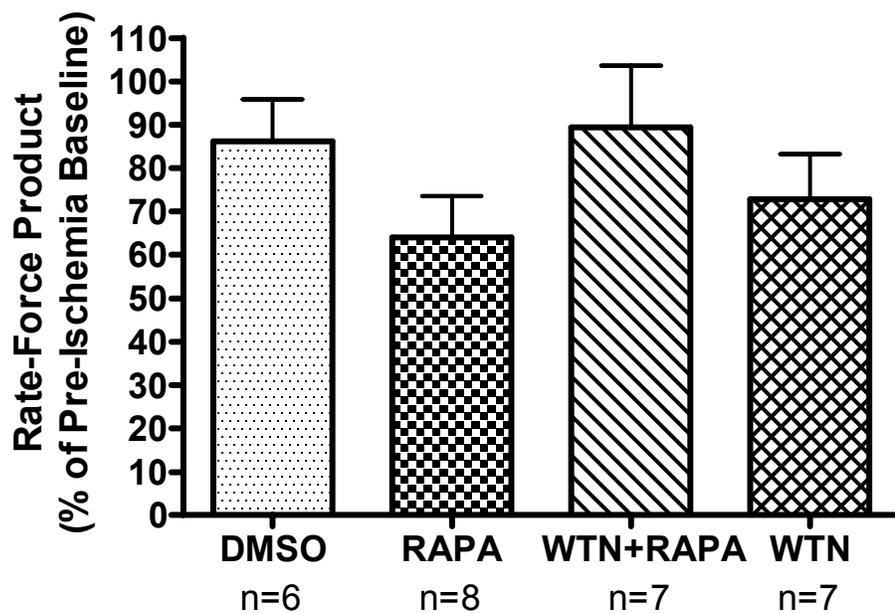
Figure 7: Rate-force product

There was no significant difference in the recovery of cardiac function (rate-force product) among groups. Rate-force product was taken as a percentage of the pre-ischemia baseline value.
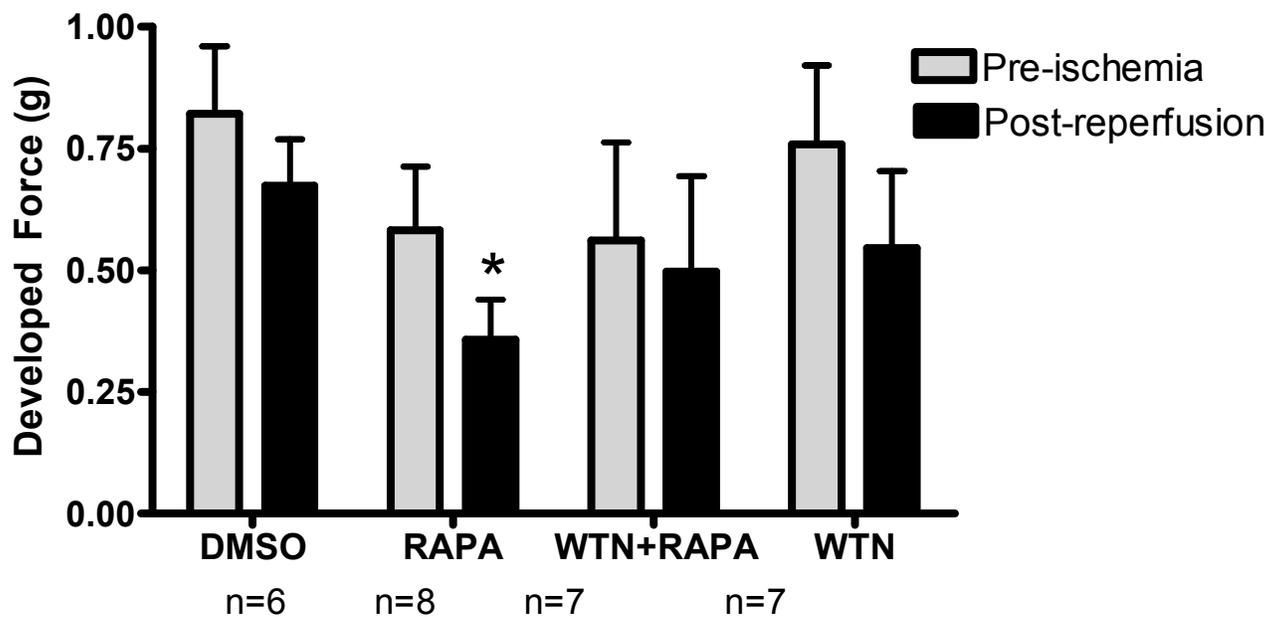
Figure 8: Developed force

Post reperfusion, a significant difference in developed force was observed between DMSO-treated and RAPA-treated groups (*P<0.05).

A
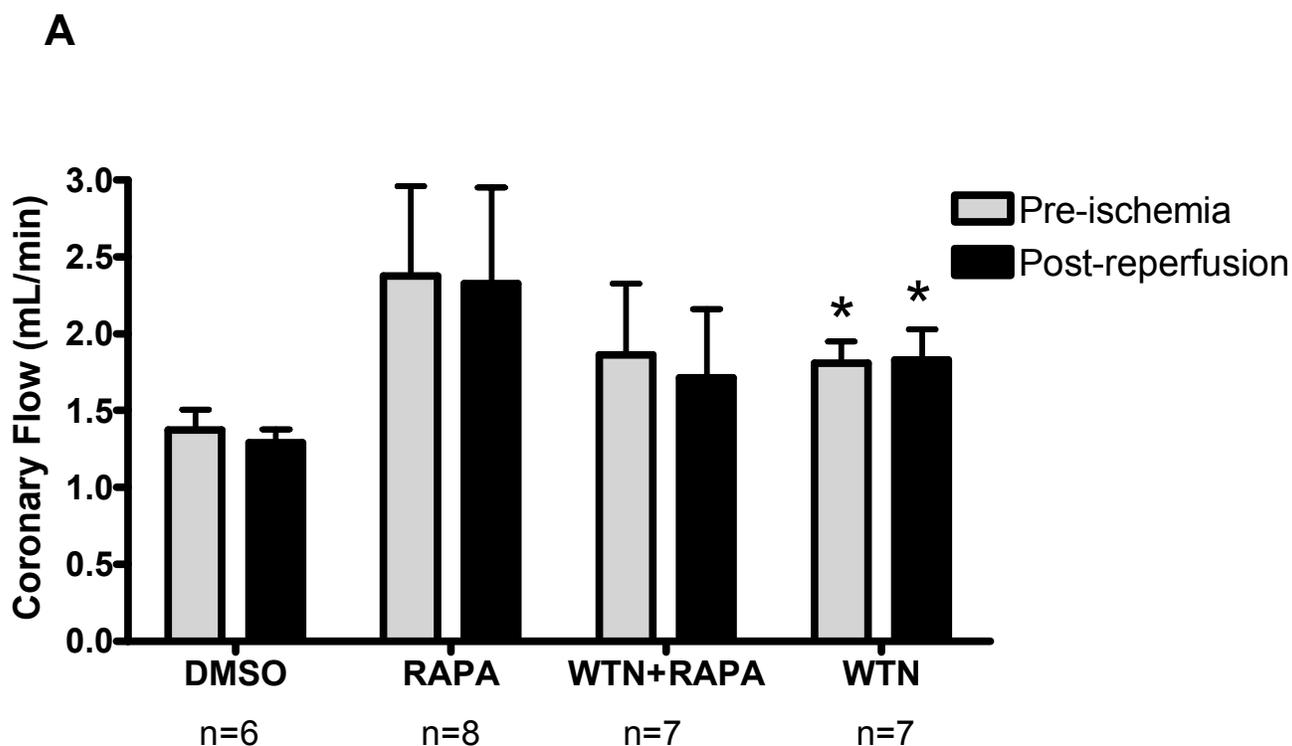
Figure 9A: Coronary flow

Mean coronary flow pre-ischemia (after 15 min stabilization and post-reperfusion (after 15 min of reperfusion). There was no significant difference in coronary flow among the groups, pre-ischemia or post-ischemia except in the group treated with WTN.

B
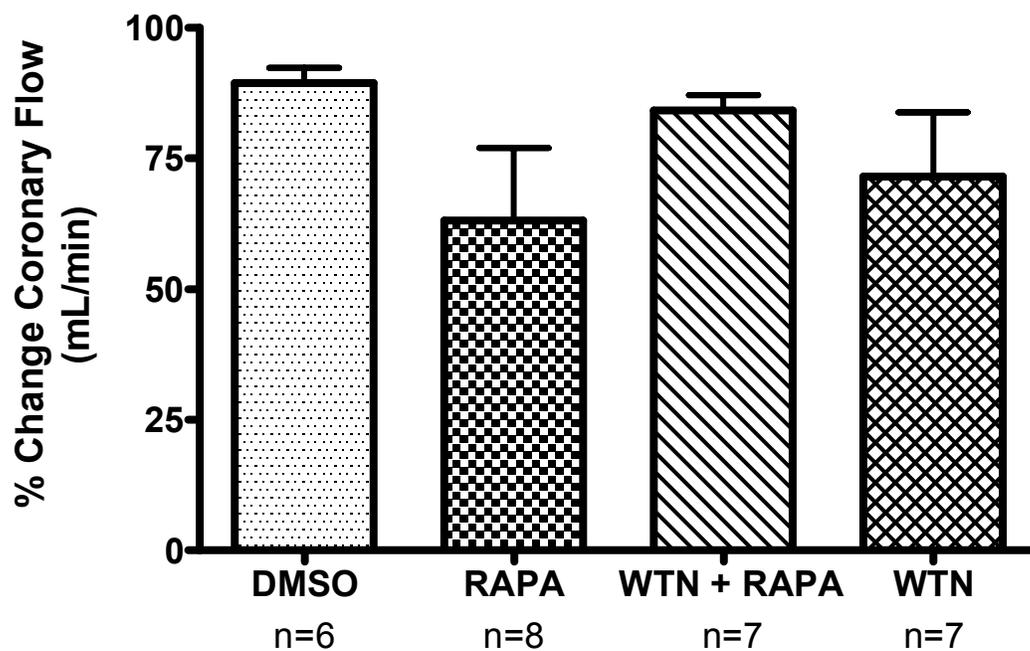
Figure 9B: Percent change of coronary flow

The percent change of coronary flow was calculated from the pre-ischemia baseline to post-reperfusion. There was no significant difference in the change in coronary flow from baseline to reperfusion among the groups. However, a marked difference can be noted between DMSO- and RAPA-treated groups.

3.5 Western blot analysis of Akt

IPC protects the heart by phosphorylating the prosurvival kinase, Akt (4). Mice were pharmacologically preconditioned with the DMSO (n=3) and rapamycin (n=3). Hearts were then harvested after 30 minutes, 1 hour, and 2 hours of pretreatment. The phosphorylation state of Akt in the prosurvival pathway was then examined via Western blot analysis. Hearts in a non-treated control group (n=2) were also blotted for changes in phosphorylation of Akt at Ser^{473}, although the DMSO-treated group was considered as the true control group as DMSO was used as the vehicle for rapamycin administration. Representative western blots are displayed in Figure 10. After careful densitometry measurement of completed Western blots, no appreciable difference in the phosphorylation state of Akt versus total Akt was observed between any of the time points or experimental groups (Figure 11). Total Akt was also blotted for although these protein levels did not vary, suggesting that any changes in kinase phosphorylation were not due to changes in total kinase levels. All in all Western blot analysis did not confirm any change in Akt phosphorylation.
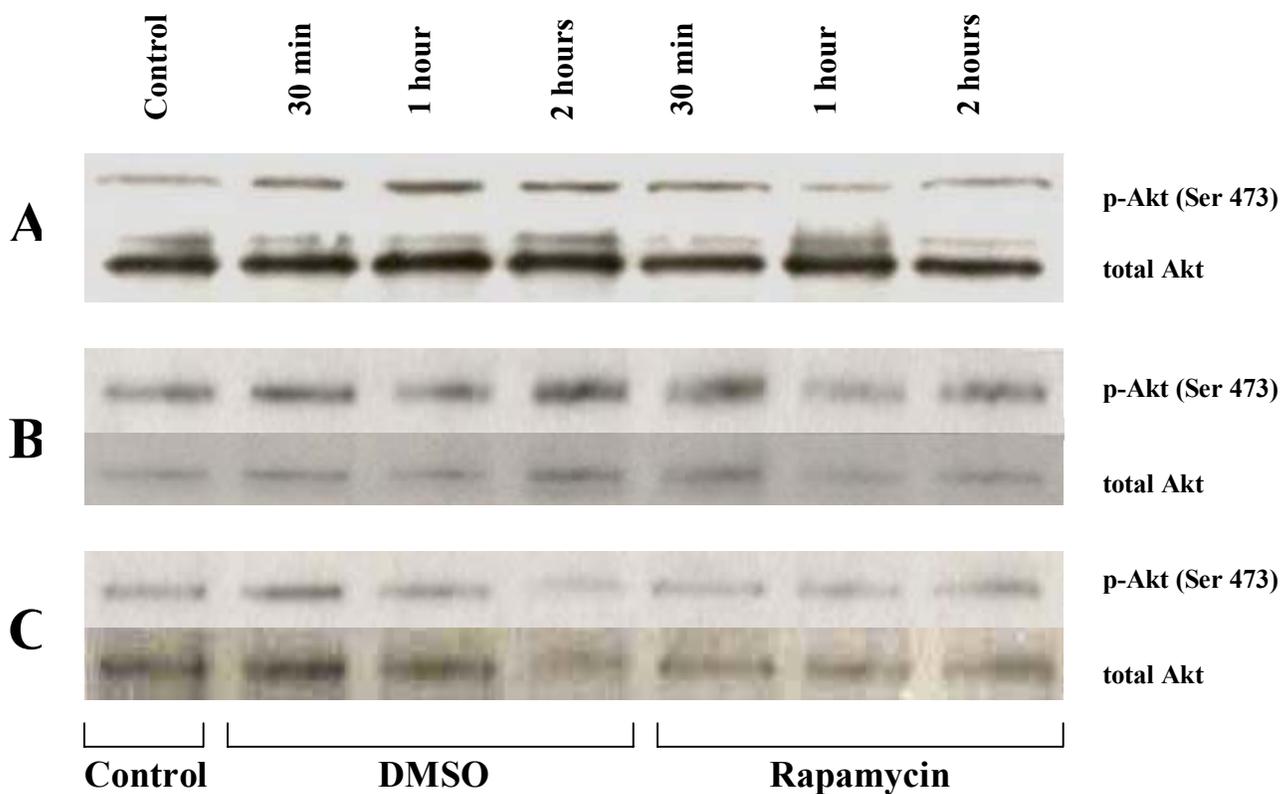
Figure 10: Western blot analysis of Akt

This analysis was performed in triplicate for DMSO- and RAPA-treated groups (A, B, C). Each Western blot is shown as a pair with phosphorylated Akt Ser[473] (top) and total Akt (bottom).
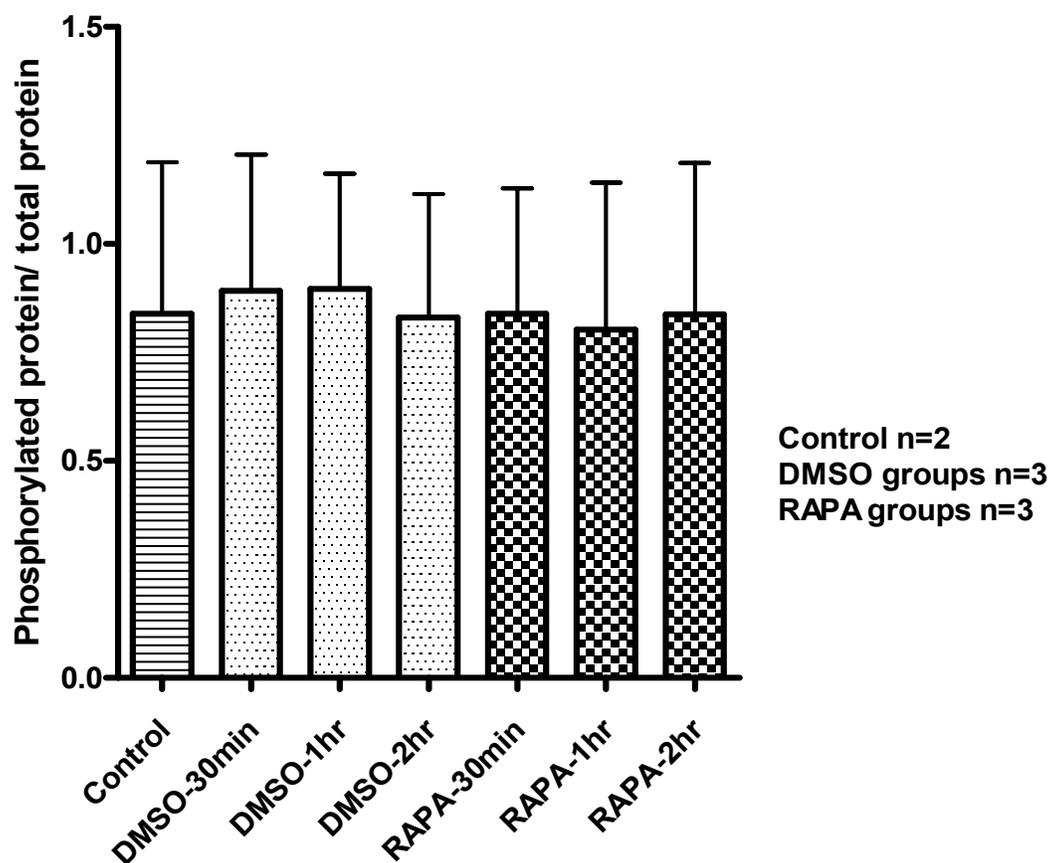
Figure 11: Western blot densitometry

Measurements were calculated as a ratio of phosphorylated Akt to total Akt which are similar among all groups and time points. The phosphorylation state of Akt was compared with densitometric analysis by the Image J software program. Bar graphs represent the mean ± SEM.

DISCUSSION

4.1 Present study

The concept that pharmacological preconditioning with rapamycin protects the heart against I-R injury was first introduced by Khan et al. in 2006 from our laboratory (25). However, the cellular and molecular mechanism that mediates this protection remains elusive. The aim of this study was to define the signaling mechanisms underlying the cardioprotective action of rapamycin. We postulated that Akt mediates rapamycin-induced protection via a positive feedback loop to Akt from mTOR, which is inhibited by rapamycin. We were particularly interested in studying the role of Akt because this signaling molecule has been shown to be activated by IPC (18; 26) and by IPC mimetics such as insulin (22) which initiate the PI3K/Akt prosurvial signaling cascade. In these studies, cardioprotection is induced through mTOR and p70S6K activation, resulting in an increase in translation and protein synthesis.

4.2 Rapamycin-induced cardioprotection

In the current investigation, mice were pre-treated with rapamycin (0.25mg/kg) after which hearts were subjected to ischemia and reperfusion in the isolated perfused heart model. The rationale for using the isolated heart model was to eliminate all circulating factors that exist *in vivo* and demonstrate the direct effect of rapamycin on the

post-ischemic recovery of function and infarct size in the intact heart. Our results show that rapamycin significantly reduced infarct size as compared to DMSO-treated control hearts. However, there was no improvement in the recovery of heart function or developed force (heart contractility), which essentially confirms the data from our previous study (25). Contrarily, we observed an increase in coronary flow in rapamycin-treated mice before ischemia and after reperfusion, which indicates a possible vasodilating property of rapamycin.

Rapamycin is a well-known inhibitor of mTOR and is usually used to block signaling in the prosurvival pathway, which abolishes cardioprotection (16; 22). This property of rapamycin presents a challenge in explaining why the current study contradicts precedent research. First, the inhibition of mTOR by rapamycin results in decreased protein synthesis, one of the highest ATP-consuming cellular processes. This could potentially direct more of the cell's energy reserve and resources to protecting the heart against ATP-depletion during ischemia. In accordance with this concept, Granville et al. showed that inhibition of mitochondrial protein synthesis with chloramphenicol in isolated perfused rat hearts reduced infarct size (12). Protection may also be animal model specific, but little additional research has been done with rapamycin and the isolated perfused mouse model to prove this conclusion.

4.3 Rapamycin-induced cardioprotection is PI3K-dependent and wortmannin alone leads cardioprotection

In the present study, wortmannin blocked cardioprotection conferred by rapamycin as demonstrated by a similar degree of infarct size as the DMSO control group. This suggests that PI3K is involved in mediating rapamycin-induced signaling. An

unexpected yet tentative finding in the current study was that wortmannin alone conferred protection in the heart against ischemia-reperfusion as demonstrated by a significant decrease in infarct size (compared to the DMSO control group). The exact mechanism of this protection is not clear although it can be explained by known actions of wortmannin. As a potent and widely used PI3K inhibitor (44), wortmannin blocks all downstream signaling targets of this protein. For example, it has been previously shown that wortmannin directly inhibits signaling functions of mTOR by irreversibly inhibiting its autokinase activity (4). Moreover, varying levels of mTOR activation in a lymphoma cell line was associated with different concentrations of wortmannin treatment. The mTOR signaling was attenuated when cells were exposed to sub- or low- micromolar concentrations of wortmannin (4). Because of the attenuated mTOR signaling as a result of wortmannin treatment, there may be reduced protein synthesis similar to rapamycin treatment, which could potentially lead to more focused activation of cell survival machinery.

Also, the specificity of wortmannin solely as a PI3K inhibitor must be questioned. Research by Davies et al. demonstrated wortmannin inhibition of other members of the PI3K superfamily in addition to PI3K itself (9). Wortmannin was found to be an inhibitor of SmLCK or smooth muscle myosin light chain kinase (37), although to a lesser degree than PI3K. Wortmannin also inhibited GSK3β also to a significant yet lesser degree than PI3K (8). Thus the protection against ischemia-reperfusion by wortmannin observed in the present study could by an inadvertent result of the inhibition of other kinases besides PI3K.

Lastly, the Ras-Raf-MEK-Erk signaling cascade has been shown to be inhibited by the PI3K/Akt signaling cascade with specific cross-talk between Akt inhibiting Raf-1 (Figure 12) (35; 45). When PI3K was blocked by wortmannin, Akt signaling was blocked as well, including inhibition of Raf-1. This could have liberated the Ras-Raf-MEK-Erk pathway to confer cardioprotection. Although there is no clear evidence available in the literature about the role of wortmannin as being cardioprotective, this leaves an area of research open to further studies.

4.4 Akt not upregulated by rapamycin

Previous studies have shown increased Akt phosphorylation after IPC. The overexpression of constitutively active Akt was discovered to reduce infarct size and apoptosis (26). Also, Li et al. reported that rapamycin significantly increased Akt phosphorylation in cardiomyocytes (30). Because of the anti-necrotic and anti-apoptotic effect of rapamycin in adult cardiomyocytes (25), we postulated that the drug may exert such cardioprotective effects through upregulation of Akt. In the present study, we monitored phosphorylation of Akt following treatment with rapamycin. Surprisingly, Akt was not phosphorylated significantly in both DMSO control and rapamycin-treated hearts. We evaluated the phosphorylation states of Akt at Ser ^{473} after 30 minutes, 1 hour and 2 hours following rapamycin treatment in order to carefully track the phosphorylation time-course of this signaling molecule. Therefore, it is unlikely that this treatment protocol might have missed the transient activation of Akt unless it occurred in less than 30 minutes after treatment with rapamycin. Another possibility is that Akt may not be upregulated at all by rapamycin without an ischemic trigger.

Finally, as mentioned earlier there is a possibility that Akt may work in concert with Erk in a parallel pathway to reach the "threshold" of mTOR or p70S6K inactivation/activation by rapamycin. Hausenloy et al. originally reported that, the PI3K/Akt and MEK-Erk signaling cascades exhibit cross-talk when they are activated by IPC. When Akt signaling was inhibited by the PI3K inhibitor LY294002, Erk in the other cascade pathway was activated. In turn, Akt was activated when PD98059 was used to inhibit the MEK-Erk pathway (17-19). In response to I-R injury, these kinase cascades activate protection through their anti-apoptotic effectors. Erk activation was also shown to be insensitive to inhibitors of PI3K such as wortmannin (9). Further evaluation on the effect of rapamycin on phosphorylation of Erk and MEK are needed to support this argument.

It is also possible that rapamycin might have another intracellular target that mediates its cardioprotection besides mTOR. Because wortmannin blocked rapamycin protection and Akt phosphorylation was not influenced by rapamycin, it can be implied that PI3K is involved in mediating signaling and not necessarily Akt. Hypothetically, rapamycin could directly target PI3K and whatever lies downstream of PI3K other than Akt for example, through a PDK1-PKC-dependent pathway. As a result, PI3K might have a direct signaling link to the Ras-Raf-MEK-Erk pathway. Rapamycin is known to inhibit mTOR and attenuate protein synthesis leaving more energy for prosurvival. Contrary to our previous hypothesis of a feedback loop between mTOR and Akt, there may be a signaling loop between mTOR and PI3K instead (see Figure 12).

4.5 Future studies

Obviously, more thorough investigations are required to demonstrate the effect of rapamycin on Akt phosphorylation. This will be accomplished by monitoring Akt phosphorylation on hearts preconditioned with rapamycin and subjected to ischemia-reperfusion in the isolated perfused heart model. In the present investigation, we were interested in studying the role of rapamycin in Akt phosphorylation without the confounding effect of ischemia-reperfusion. Other kinases in PI3K/Akt must also be blotted for such as PDK1, mTOR, and p70S6K along with kinases in the prosurvival pathways such as Erk and Raf also need to be investigated to see if they have a role in cardioprotection with rapamycin.

4.6 Conclusions

Although preventive medicine will most effectively control the prevalence of heart disease and myocardial infarctions, limitations such as the expenses and availability of medicine impairs this approach. Novel medical treatments that will limit the extent of damage caused by a myocardial infarction are urgently needed. Cellular protection or tolerance against ischemia is coming to the forefront of research in order to find new methods for treating cardiovascular diseases. This study acknowledges rapamycin as an important pharmacological agent that protects the heart against I-R injury. To our knowledge this is the first study implicating divergent roles of PI3K and Akt in the signaling mechanism of rapamycin-induced cardioprotection in the isolated perfused mouse model. Development of this model lays the groundwork for investigating other signaling pathways for the benefit and management of acute myocardial infarction.

Figure 12: Final hypothetical signaling pathway
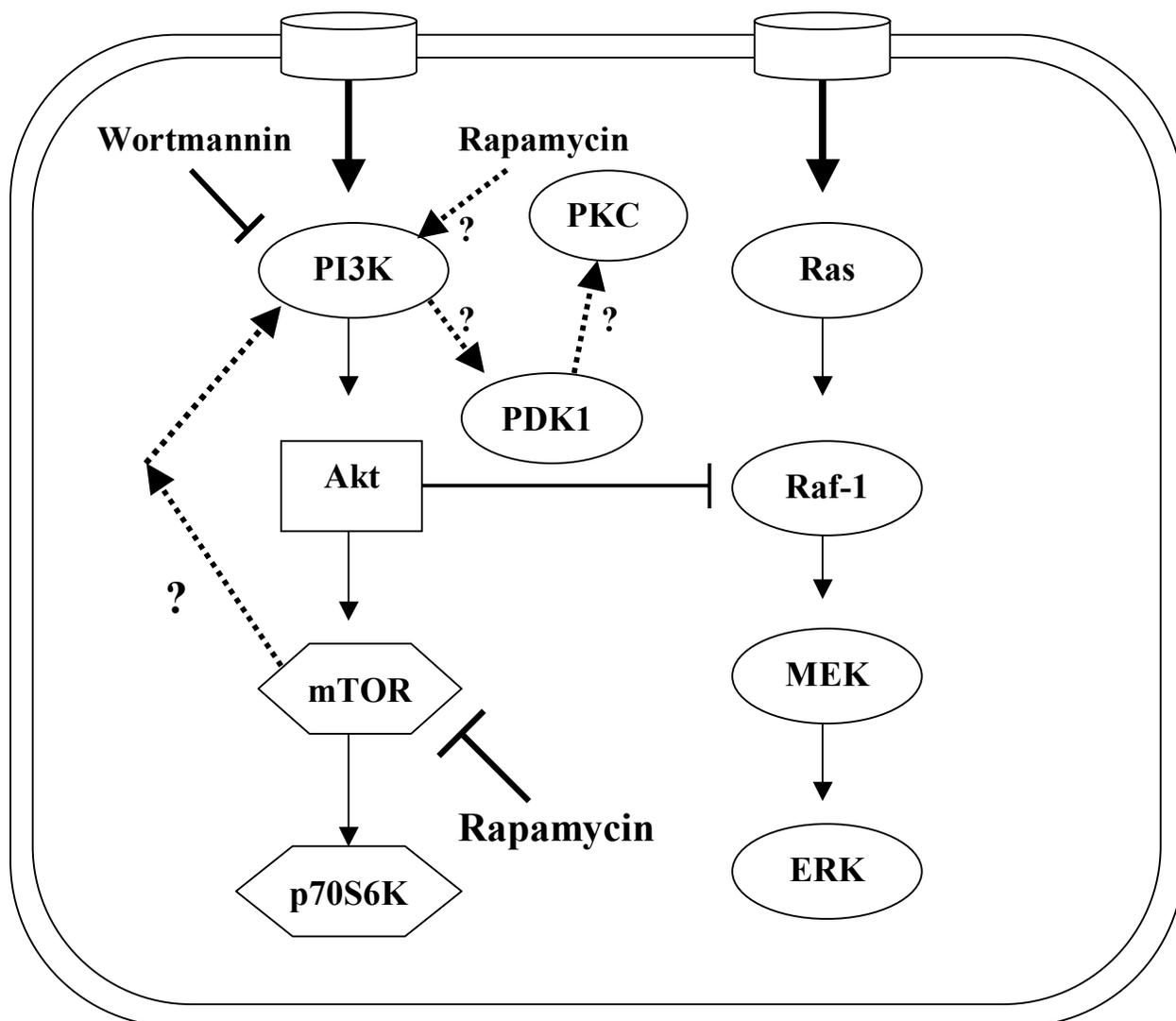
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VITA

Shivani Kirit Desai was born on July 8, 1984, in London, England and is a citizen of the United States of America. She graduated from Mills E. Godwin High School in Richmond, Virginia in 2001. Shivani received a Bachelor of Science in Biology from The College of William and Mary in May of 2005. She continued on to get a Master of Science in Physiology at the Virginia Commonwealth University School of Medicine. While pursuing her Masters degree, she worked as a teaching assistant of undergraduate physiology and served as secretary of the Physiology Graduate Student Association. Shivani plans to attend medical school in 2008.