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A NOVEL ROLE OF SIRT1 IN SILDENAFIL INDUCED CARDIOPROTECTION IN MICE

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A NOVEL ROLE OF SIRT1 IN SILDENAFIL INDUCED CARDIOPROTECTION IN MICE

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

by

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List of Abbreviations

AMI	Acute Myocardial Ischemia
A.U.	Arbitrary units
AWDT	Anterior wall diastolic thickness
AWST	Anterior wall systolic thickness
cGMP	Cyclic guanine monophosphate
DMSO	Dimethyl sulfoxide
EF	Ejection fraction
eNOS	Endothelial nitric oxide synthase
FS	Fractional shortening
GC	Guanylyl cyclase
H	Hour
HDAC	Histone deacytylase
HR	Heart rate
HSF	Heat shock factor
HSP	Heat shock protein
HSR	Heat shock response
I-R	Ischemia-Reperfusion
IP	Ischemic Preconditioning
ip	Intraperitoneal
iNOS	Inducible nitric oxide synthase
LAD	Left anterior decending coronary artery
LV	Left ventricle
LVEDD	Left ventricular end-diastolic diameter
LVESD	Left ventricular end-systolic diameter
Min	Minutes
NAD	Nicotinamide adnenine dinucleotide
NO	Nitric oxide
PBS	Phosphate buffered saline
PDE-5	Phosphodiesterase 5
PGC-1α	Peroxisome proliferation activating receptor-gamma-co-activator-1-alpha
PKG	Protein kinase G
PWDT	Posterior wall diastolic thickness
PWST	Posterior wall systolic thickness
ROS	Reactive oxygen species
RSV	Resveratrol
SIL	Sildenafil
TSA	Trichostatin A
TTC	2, 3, 5-triphenyl tetrazolium chloride

ABSTRACT

A NOVEL ROLE OF SIRT1 IN SILDENAFIL INDUCED CARDIOPROTECTION IN MICE

By Mona B. Shalwala, B.S.E.

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

Virginia Commonwealth University, 2010

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Phosphodiesterase-5 inhibitor, sildenafil (SIL) protects against myocardial ischemia/reperfusion (I-R) injury. We hypothesized that SIL-induced protection may be mediated through activation of SIRT1, an enzyme which deacetylates proteins involved in cellular stress response. Adult male ICR mice were treated with SIL (0.7mg/kg ip), Resveratrol (RSV) (5mg/kg ip) (positive control), or saline (0.2 ml ip). The hearts were

harvested 24 h later and homogenized for SIRT1 activity analysis. Both SIL and RSV increased cardiac SIRT1 activity ($P<0.001$) as compared to Saline. Adult mouse ventricular cardiomyocytes pre-treated with either SIL or RSV (1μM) *in vitro* also upregulated SIRT1 activity ($P<0.05$). SIL also reduced infarct size following 30 min. ischemia and 24 h reperfusion in vivo. Sirtinol (5mg/kg in 10% DMSO, ip), a SIRT1 inhibitor abolished the infarct-limiting effect of SIL and RSV ($P<0.001$). In conclusion, activation of SIRT1 by SIL plays an essential role in cardioprotection against I-R injury.

INTRODUCTION

1.1 Cardiovascular Disease and Myocardial Ischemia/Reperfusion Injury:

According to the World Health Organization, cardiovascular disease ranks number one among the causes of death and is projected to remain the leading cause of death worldwide. Cardiovascular disease accounts for approximately 1 million deaths in the United States annually. There are an estimated 62 million people with cardiovascular disease and 50 million people with hypertension in this country.[1] The greatest affected population is over the age of 65. It is expected that as the population continues to age, and life expectancy continues to increase, there will be a growing population of people afflicted with heart conditions requiring costly health care interventions such as coronary bypass graft surgery and angioplasty.[2] Heart related conditions can lead to other organ damage, and can become very costly and debilitating for the patient.

Acute myocardial ischemia (AMI) is a cardiovascular disease that affects an estimated 1.5 million people in the United States annually and is a major cause of mortality and morbidity worldwide. Over half of the patients with AMI die within one hour of the event. AMI occurs when there is a sudden occlusion of an artery supplying blood to myocardium, depriving the area of blood, oxygen and nutrients, and causing dramatic changes in the cellular metabolism and function within the tissue. Myocardial infarction can cause severe cellular and tissue damage such as myocyte hypertrophy, myocyte death,

and disruption of matrix metalloproteinase balance. Following myocardial ischemia, there is a loss of contracting myocardium and a resulting increase in the workload on the viable myocardium. The cardiac overload caused by myocardial infarction increases energy utilization and supply-demand imbalance, which lead to cellular necrosis and apoptosis. This subsequently leads to remodeling and worsening of left ventricular function, and eventually heart failure.[3]

1.2 Ischemic and Pharmacological Preconditioning:

Ischemic Preconditioning (IP) is an innate mechanism used to protect against I-R injury by simulating angina in the clinical setting. IP was first documented in 1986 after experiments were conducted in a canine heart by Murry et. al. It was found that brief episodes of ischemia can protect the heart from a subsequent prolonged ischemic episode.[10, 11] [12, 13] IP has been shown to reduce myocardial infarct size as well as the extent of damage to skeletal muscle, brain or hepatic tissue induced by subsequent exposure to severe ischemia in a variety of species.[10, 12]

Two different phases of IP cardioprotection have been observed, the early phase and the late phase. The early phase is referred to as "classic" preconditioning which is observed immediately after brief periods of ischemia and lasts for approximately 1-2 hours.[10, 11] The second phase is referred to as the "delayed" phase of preconditioning, and is observed at 12 hours following a brief ischemic insult and lasts up to 96 hours.[10, 11] The late phase of preconditioning has been the focus of most studies because of the potential to exploit the protective mechanisms against ischemic heart disease in patients.[2, 13]

Several intracellular signaling pathways and receptors have been found to be involved in the cardioprotective effect of preconditioning. Endogenous mediators such as adenosine, catecholamines such as norepinephrine, opiods, free radicals, and bradykinin have all been shown to trigger preconditioning.[8, 11] Proposed IP mechanisms include the release of these endogenous mediators, activation of G-coupled receptors and protein kinase C, the opening of mitochondrial K_{ATP} channels, and synthesis of cytoprotective proteins including endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), cyclooxygenase-2, and heat shock protein HSP70.[12]

Pharmacological activators of the pathways involved in IP have been given exogenously and have been shown to exhibit cardioprotective effects as well. However, few agents have been implemented in the clinical setting against ischemic heart disease.[2] Over the last 8 years, our laboratory has pioneered the concept that potent phosphodiesterase-5 (PDE-5) inhibitors, including Sildenafil (Viagra), Vardenafil (Levitra) and Tadalafil (Cialis), which are known to enhance erectile function in men induce powerful cardioprotective effect against I-R injury in the rabbit and mouse hearts[21,48],myocardial ischemia-induced heart failure[3] and doxorubicin-induced cardiomyopathy in mice.[49] We also deomonstrated that the protective effect of SIL was dependent on the opening of ATP-sensitive mitochondrial (mitoK_{ATP}) channels. Our hypothesis was that potent vasodilatory action of SIL (and other PDE-5 inhibitors) could potentially release endogenous mediators of IP including adenosine[6], that may trigger receptor-mediated signaling cascade (through the action of kinases)[9] resulting in phosphorylation of eNOS and release of NO.[46] The generation of NO could potentially

activate guanylyl cyclase (GC) leading to enhanced formation of cGMP. cGMP further activate protein kinase G (PKG) that can subsequently open mitoK_{ATP} channel resulting in cardioprotective effects.[50]

1.3 SIRT1:

In recent years, histone deacetylases (HDACs) have been shown to regulate cardiac hypertrophy and other cardiac diseases.[33] There are three classes of HDACs that have been recognized in humans: class I and class II which are zinc-dependent amidohydrolases, and class III which are nicotinamide adenine dinucleotide (NAD+) -dependent.[33] NAD+-dependent deacetylases are also termed as sirtuins due to their homology with the yeast histone deacetylase Sir2.[33] SIRT1 is part of the class III Sirtuin family of HDACs. SIRT1 catalyzes a reaction in which nicotinamide is liberated from NAD+ and the acetyl group of the substrate is transferred to cleaved NAD+, generating the novel metabolite O-acetyl-ADP ribose.[32, 34]

Since SIRT1 can deacetylate a variety of substrates, it is involved in a broad range of physiological functions. The murine homolog of SIRT1 is Sir2.[28] Mice deficient in Sir2 exhibit developmental abnormality in the heart and rarely survive postnatally, suggesting that Sir2 has important functions in the heart.[28] Sirtuins have also been linked to cancer, muscle differentiation, inflammation, obesity, and neurodegeneration.[4, 32] SIRT1 has anti-aging effects, and has been shown to extend the life span in yeast and worms.[32] SIRT1 has many proven biological functions including control of gene expression, cell cycle regulation, apoptosis, DNA repair, metabolism, oxidative stress

response and aging.[35] SIRT1 activity has also been correlated with metabolic state of cells and it is believed to be nutrient regulated due in part to its dependency on NAD.[32, 34, 36-38] Unlike class I and II HDACs, SIRT1 deacetylates non-histone proteins such as Forkhead transcription factors (FOXO), peroxisome proliferation activating receptor (PPAR)-gamma co-activator-1α (PGC-1α), nuclear factor κB, myoD, ku70, and p53.[27, 29, 30, 34]

1.4 Activator and Inhibitor of SIRT1:

Resveratrol (RSV) is the best naturally occurring putative small molecule activator of SIRT1[27-30], which allosterically binds to SIRT1 and lowers the Michaelis constant of SIRT1 for both the acetylated substrate and NAD+.[31, 32] RSV has been shown to be a pharmacological preconditioning agent which mimics ischemic preconditioning like effect in the kidney, heart, and brain against ischemic injury, however the mechanism of action remains unclear.

Sirtinol is a putative inhibitor of SIRT1 and has been used as such extensively in literature.[29, 32, 33] The aromatic ring moieties of the inhibitor interact with the hydrophobic potions of the residues Thr89, Lys287, Glu323, and Cys324 on SIRT1, which normally functions as the binding site for the adenine base of NAD+.[33] When Sirtinol interacts with SIRT1, it prevents NAD+ from reacting thereby inhibiting SIRT1 functionality.

1.5 SIRT1 and Cellular Stress Response:

Stress resistance is a measure of the cells ability to survive under detrimental conditions. A number of proteins have been implicated in the stress response, including SIRT1.

'Hormesis' is the phenomenon in which a mild stress, such as IP, can induce a protective response against subsequent stresses. This response is credited for how mildly stressed animals paradoxically outlive their unstressed peers.[26] This has been demonstrated in calorie-restricted yeast, where mildly nutrient deprived yeast survived longer than the control.[26] Caloric restriction is defined as a reduction of calorie intake, typically by 30–40% in rodents, to a level that does not cause malnutrition and increases lifespan and stress resistance in multiple species.[26] SIRT1 expression is upregulated in calorie restricted mice and 3- to 9-fold in response to cardiac stress in monkeys.[35] The balance between growth and death in cardiac myocytes plays an important role in determining the pathophysiology of cardiac diseases.[35] Cardiac myocytes can be transcriptionally reprogrammed in response to cellular stress. Studies have shown antioxidants and heat shock protein expression increases as a cell protective mechanism.[35] The amount of SIRT1 expression and activity upregulation is a compensatory mechanism, and has been shown to retard aging and inhibit apoptosis without causing NAD+ depletion or mitochondrial dysfunction.[35] Research has shown that SIRT1 has been associated with RSV's cardioprotective effect on myocyte survival following ischemia.[30] SIRT1 expression is upregulated in response to proapoptotic stimuli.[28] The mechanism by which SIRT1 is believed to protect cardiomyocytes from apoptosis is through inactivation of p53 by deacyetylation.[39] SIRT1 is upregulated in response to stress in the heart such as pressure overload and paraquat injections which induce oxidative stress.[35] SIRT1 upregulation has also been correlated with age, older monkey hearts had almost three times as much SIRT1 upregulated when compared to young monkey hearts.[35]

SIRT1 is an important regulator of cell defenses and survival in response to stress.[30, 32] SIRT1 represses p53-dependent apoptosis in response to oxidative stress and DNA damage.[30] FOXO3 is a transcription factor deacetylated by SIRT1, that regulates several genes involved in reactive oxygen species (ROS) detoxification, cell cycle arrest (Cyclin D2), apoptosis, and DNA damage repair. FOXO3a acts as a cellular stress sensor and mediates the stress response.[16, 24] Studies have shown that in response to oxidative stress, SIRT1 and FOXO3 form a complex and the ability of FOXO3 to induce DNA damage repair and cell cycle arrest is enhanced.[16, 24, 30] SIRT1 provides protection against apoptosis and has an essential role in mediating the survival of neurons and cardiac myocytes under stress in vitro.[28] There is evidence that inhibition of SIRT1 activity in cardiomyocytes leads to an increase in the basal rate of apoptosis and upregulation of hypertrophy associated genes.[4, 39]

1.6 PGC-1α and Myocardial I-R Injury:

Mitochondria are the main energy sources of cells, and compromised mitochondrial function can lead to cardiovascular and metabolic diseases.[25] Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha, also known as PPARGC1A and PGC-1α, is a gene that encodes for a gene that is a transcriptional coactivator that regulates transcription of genes involved in a wide variety of biological responses including mitochondrial biogenesis, adaptive thermogenesis, glucose/fatty acid metabolism, fiber type switching in skeletal muscle, and heart development.[25, 34] Since PGC-1α controls mitochondrial biogenesis and function, it plays an important role in cell survival. PGC-1α

may also play a key role in the attenuation of I-R injury since ischemia causes sequential changes in mitochondrial morphology in the myocardium. Increased expression of PCG-1α has been shown to increase mitochondrial biogenesis and oxidative metabolism. When there is an increased demand for ATP and damage to mitochondrial integrity, it is expected that the expression of PGC-1α will also increase.

MitoK_{ATP} channels act as metabolic sensors that regulate cellular activity according to cellular energetic demands, and the opening of these channels is necessary for the infarct-sparing effects of preconditioning.[13, 42] Regulating mitochondrial membrane potential is a crucial component to cellular stress response.[34] Upon a stress stimuli, PGC-1α transiently enters the nucleus to activate transcription of target genes, and then PGC-1α is either degraded in the nucleus or returns to the cytoplasm.[34] PGC-1α has been shown to be required for the adaptation to the stress-induced change in mitochondrial membrane potential.[34]

1.7 SIRT1 and Heat Shock Response:

The heat shock response (HSR) is the cellular response to stress. The heat shock response was first recognized in response to elevated temperature, however it is an essential mechanism for the protection of cells in any type of cellular stress. As part of the HSR, heat shock proteins (HSPs) are induced to act as molecular chaperones to repair damaged proteins. HSP production can also be induced by non-stress biological stimuli such as growth factors and differentiation factors. Therefore, HSPs have an important role in maintaining cellular homeostasis. HSPs are a set of highly conserved proteins with

molecular masses of 28, 60, 70, 82, 90, and 110 kDa. The most abundant heat shock protein is HSP70. These proteins are typically induced by stresses such as heat shock, ischemia, exposure to transition metals, pressure overload, and exposure to oxygen radicals.[13] HSP70 has been shown to be a molecular chaperone that aids in cardioprotection against metabolic insults such as I-R injury. The amount of HSP70 is directly proportional to the amount of cardioprotection against I-R injury.[13] Studies in transgenic mice overexpressing HSP70 have shown that this protein confers protection against I-R injury.[13] Myocardial HSP70 is upregulated 24 hours after IP.[13]

Heat Shock Factor 1 (HSF1) is a gene that encodes a transcription factor that regulates gene expression in response to cellular stress, including the human HSP70 gene. Transient activation of HSF1 occurs through various environmental and physiological stresses. The activation of HSF1 is a multi-step process in which an HSF1 monomer is converted to a DNA-binding trimer and becomes phosphorylated at serine residues. For a brief time, HSF1 is capable of binding to DNA promoters and enhancing transcription.[43] In its deacetylated state, HSF1 can bind to DNA. Activation of SIRT1 has been shown to prolong HSF1 binding to the heat shock promoter of HSP70.[43] It was also shown that downregulation of SIRT1 attenuated the heat shock response (HSR), and release of HSF1 from its promoter elements.[43] Therefore, the abundance and activity of SIRT1 was shown to regulate the attenuation of the HSR.[43] SIRT1 has a central role in the maintenance of protein homeostasis and mediating the stress response.[43]

1.8 Rationale for the Present Study

The aim of this study was to determine if SIL induces protection against I-R through increased expression and activation of SIRT1. The secondary purpose was to determine the effect of SIL on eNOS, iNOS, HSP70, HSF1, and PGC-1α expression. We hypothesized that SIRT1 expression and activity is necessary for the cardioprotective effects of SIL due to the overlap of signaling pathways involved the cellular response to I-R injury. RSV, a known pharmacological preconditioning agent, is one of the most potent small molecule activators of SIRT1, and was used as a positive control in all of the studies. If SIL increases SIRT1 activity and/or expression, it may indicate that SIL has other possible therapeutic benefits.

To test this hypothesis we used a murine model of I-R where we occluded left coronary artery for 30 minutes and reperfused the heart for 24 hours. The effect of treatment with SIL was compared to that with RSV and a Saline control. Sirtinol was administered with SIL to determine if the use of a SIRT1 inhibitor would abolish protein expression, protein activity, and cardiac remodeling normally present upon treatment with SIL alone.

MATERIALS AND METHODS

2.1 Animals:

Adult male ICR mice weighing 35.5 ± 5g 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). All experimental preparations and protocols involving animals were reviewed and approved by the Animal Care and Use Committee of Virginia Commonwealth University.

2.2 Antibodies and Chemicals:

SIRT1 (Sigma-Aldrich, St. Louis, MO), HSP70 (Santa Cruz Biotechnology), PGC-1α (GenWay, San Diego), HSF1 (Santa Cruz Biotechnology), eNOS (Santa Cruz Biotechnology), iNOS (Santa Cruz Biotechnology), donkey anti-goat IgG-HRP (Santa Cruz Biotechnology), donkey anti-rabbit IgG (Amersham) and β-Actin (Santa Cruz Biotechnology) were used for Western blots. SIRT1 (Cyclex) and Protein A agarose beads were used for Immunoprecipitation. Triphenyltetrazolium chloride (TTC) and Formalin were purchased from Sigma-Aldrich (St. Louis, MO)

2.3 Drug Preparation:

Trans-Resveratrol powder was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in 15% dimethyl sulfoxide (DMSO) and saline (0.9% NaCl containing 2.5 mM $CaCl_2$) for intraperitoneal (*ip)* injection. Sildenafil citrate powder was kindly provided by Pfizer and was dissolved in saline for *ip* injection. Sirtinol was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in 10% warm PBS and sonicated until homogenous.[44] Final concentrations of the drugs injected in ICR mice *ip* were 0.7mg/kg, 5mg/kg, and 5mg/kg for SIL, RSV, and Sirtinol respectively. The SIL dose of 0.7 mg/kg *ip* was chosen to simulate the serum level of SIL for a patient with 70 kg body weight after orally taking a l00 mg tablet of SIL.[2, 26] The therapeutic dose range for SIL is 25 to 100 mg once a day.[17] Although the plasma half-life of SIL is only 4 hours, the therapeutic benefits have been documented up to 24 hours after administration.[17] The doses of RSV and Sirtinol were determined based on a previous study where these drugs were administered *ip*.[44]

2.4 Myocardial infarction protocol:

Surgical procedures were followed as previously reported.[3] In brief, the animals were anesthetized with the injection of pentobarbital sodium (70 mg/kg ip), intubated orotracheally, and ventilated on a positive-pressure ventilator. The tidal volume was set at 0.2 ml, and the respiratory rate was adjusted to 133 cycles/min. All surgical procedures were carried out under sterile conditions. A left thoracotomy was performed at the fourth intercostal space, and the heart was exposed by stripping the pericardium. The left anterior

descending coronary artery (LAD) was then identified and occluded for 30 minutes by a 7.0 silk ligature that was placed around it with a small tube occluding flow. After 30 minutes of ischemia the tube was removed to reperfuse the tissue. The air was expelled from the chest.[3] Infarct size, protein expression, or protein activity was measured from the heart harvested after 24 h of reperfusion.

Survival. Animals that died during the surgical procedure or during recovery were excluded.

Experimental Protocol 1: Infarct Size Assessment. (See Figure 1) In these studies, SIL (0.7 mg/kg), RSV (5 mg/kg), or Saline (volume matched 0.2ml) was given *ip* in ICR mice and 24 h later, the mice were subjected to ischemia and reperfusion. In SIRT1 inhibitor studies, the ICR mice were given an initial *ip* injection of either SIL (0.7 mg/kg), RSV (5 mg/kg in 15% DMSO), or Saline (volume matched 0.2ml), and 24 h later given either Sirtinol (5 mg/kg in 10% DMSO) or 10% DMSO in warm PBS (volume matched 0.2ml) 30 minutes prior to I-R. 24 h after reperfusion Infarct size was determined. This was a single-blind study, where the surgeon was blinded to the type of drug administered to the mice.

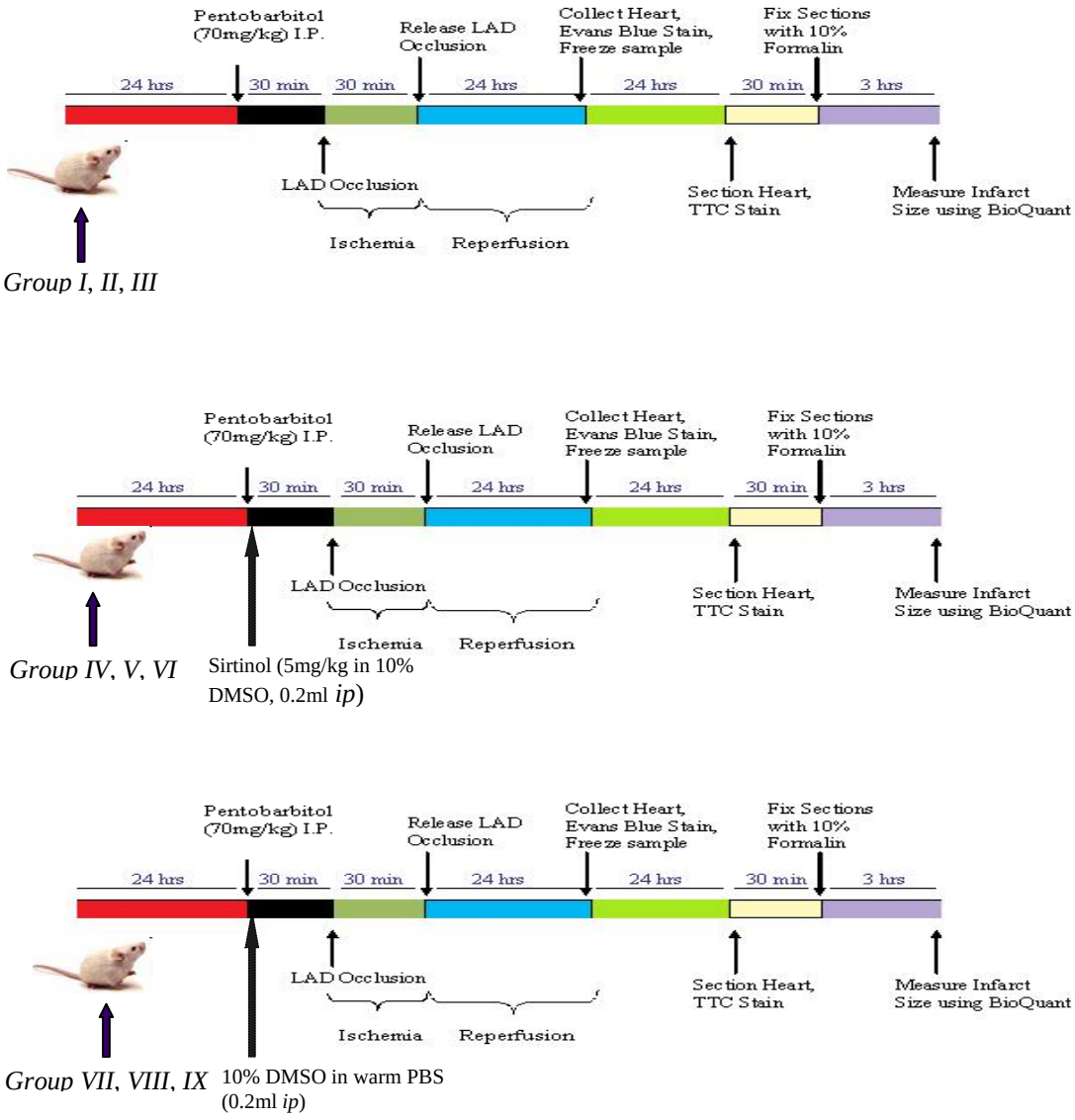


Figure 1. Experimental Protocol 1. *ip* injections were given as follows: Group I: Saline (volume matched, 0.2ml); Group II: Resveratrol (5mg/kg, 0.2ml); Group III: Sildenafil (0.7mg/kg, 0.2ml); Group IV: Saline (volume matched, 0.2ml) and Sirtinol (5 mg/kg, 0.2ml); Group V: Resveratrol (5mg/kg, 0.2ml) and Sirtinol (5 mg/kg, 0.2ml); Group VI: Sildenafil (0.7mg/kg, 0.2ml) and Sirtinol (5 mg/kg, 0.2ml); Group VII: Saline (0.2ml) and DMSO (%10 in warm PBS, 0.2ml); Group VIII: Resveratrol (5mg/kg, 0.2ml) and DMSO (%10 in warm PBS, 0.2ml); Group IX: Sildenafil (0.7mg/kg, 0.2ml) and DMSO (%10 in warm PBS, 0.2ml)

Experimental Protocol 2: Protein Expression and Activity Assessment following I-R. (See Figure 2) In these studies, SIL (0.7 mg/kg), RSV (5 mg/kg), or Saline (volume matched 0.2ml) was given *ip* in ICR mice and 24 h later the mice were subjected to 30 minutes of ischemia and 24 h of reperfusion. Sham mice were used as a control for surgical conditions, and all of the same procedures were followed except instead of ligating the LAD, a 7.0 silk suture was passed underneath the LAD and removed immediately. In SIRT1 inhibitor studies only SIRT1 activity was measured, the ICR mice were given an initial *ip* injection of either SIL (0.7 mg/kg), RSV (5 mg/kg in 15% DMSO), or Saline (volume matched 0.2ml), and 24 h later given either Sirtinol (5 mg/kg in 10% DMSO) or 10% DMSO in warm PBS (volume matched 0.2ml) 30 minutes prior to I-R. The mice were anesthetized with the injection of pentobarbital sodium (70 mg/kg ip), their hearts were removed, and frozen at -80 degrees Celsius until use.

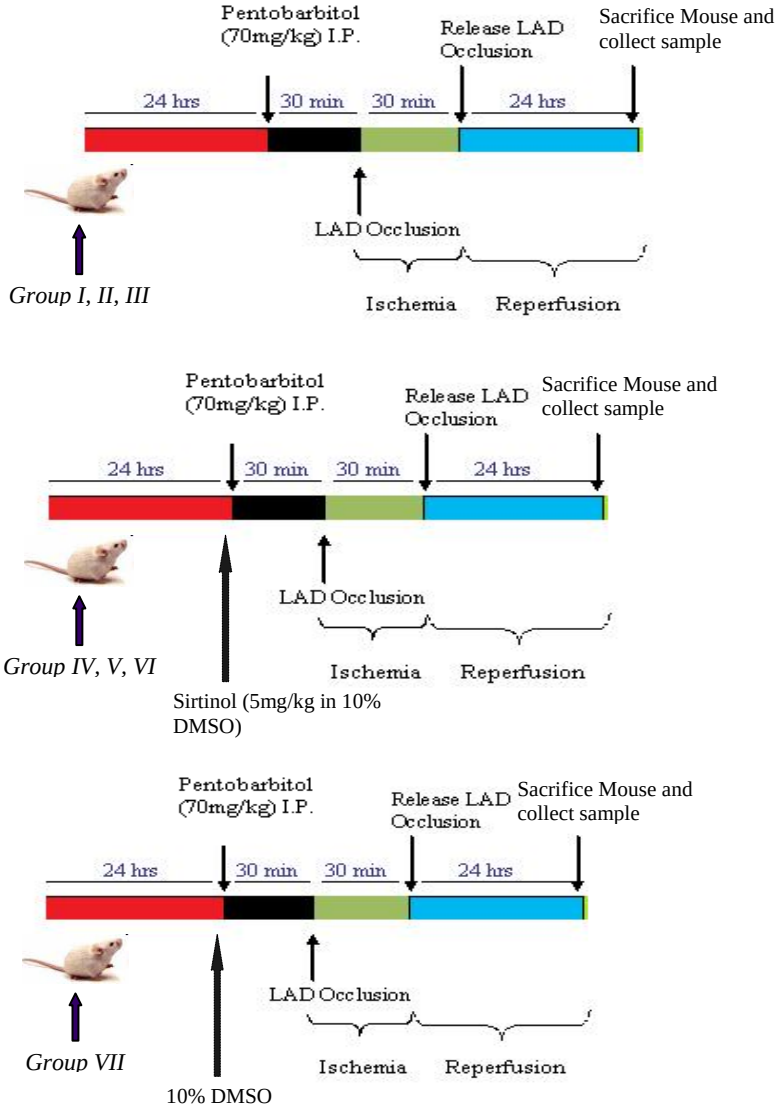


Figure 2. Experimental Protocol 2. *ip* injections were given as follows: Group I: Saline (volume matched, 0.2ml); Group II: Resveratrol (5mg/kg, 0.2ml); Group III: Sildenafil (0.7mg/kg, 0.2ml); Group IV: Saline (volume matched, 0.2ml) and Sirtinol (5 mg/kg, 0.2ml); Group V: Resveratrol (5mg/kg, 0.2ml) and Sirtinol (5 mg/kg, 0.2ml); Group VI: Sildenafil (0.7mg/kg, 0.2ml) and Sirtinol (5 mg/kg, 0.2ml); Group VII: Saline (0.2ml) and DMSO (%10 in warm PBS, 0.2ml) The mice were anesthetized with the injection of pentobarbital sodium (70 mg/kg ip), their hearts were removed, and frozen at -80 degrees Celsius until use.

2.5 Measurement of Infarct Size and Risk Area:

Infarct Size and Risk Area were determined using methods similar to those previously reported.[3, 45] At the end of reperfusion, the mouse was anesthetized with the injection of pentobarbital sodium (70 mg/kg ip). The heart was excised from the mouse and mounted on a Langendorff apparatus. The coronary arteries were perfused with 0.9% NaCl containing 2.5 mM $CaCl_2$. After the blood was washed out, 2 ml of 10% Evans blue dye were injected as a bolus into the aorta until most of the heart turned blue. The heart was perfused with saline to wash out the excess Evans blue. The stained heart was removed from the Langendorff apparatus and frozen at -20 °C. The frozen heart was cut into six to eight transverse slices. 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). The infarct area and risk area was measured using computer morphometry (Bioquant 98). The risk area was calculated as total ventricular area minus the area of the cavities. The infarct size was presented as percentage of the risk area (See Figure 3).

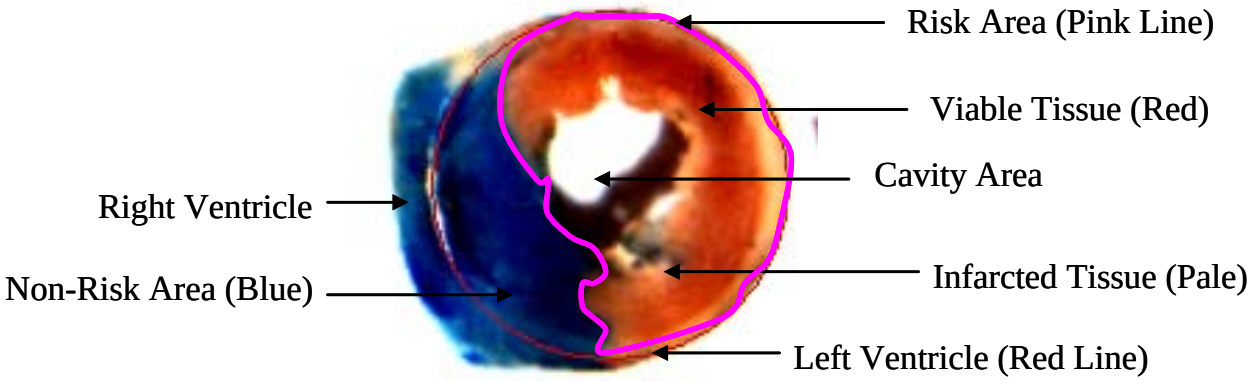


Figure 3. Representative section of mouse heart. This cross section shows the criteria used to determine the risk area, non-risk area, infracted tissue, cavity area, and viable tissue. The risk area was calculated as total ventricular area minus the area of the cavities. The infarct size was presented as percentage of the risk area.

2.6 Assessment of Heart Function:

Doppler Echocardiography was performed using the Vevo770^{TM} imaging system (VisualSonics Inc., Toronto, Canada) prior to surgery (baseline), and after 24 hours of reperfusion using techniques similar to those previously reported.[3] Mice were anesthetized with pentobarbital sodium (30 mg/kg ip). The mice were placed in the supine position, and ECG limb electrodes were attached. The chest was carefully shaved, and ultrasound gel was used on the thorax to optimize visibility during the exam. A 30-MHz probe was used to obtain two-dimensional (B-Mode), and cross-sectional (M-mode) imaging from parasternal short-axis view at the level of the papillary muscles. M-mode images of the LV were obtained, and systolic and diastolic wall thickness (anterior and posterior) and LV end-systolic and end-diastolic diameters (LVESD and LVEDD, respectively), Ejection Fraction (EF), Fractional Shortening (FS), and heart rate (HR) were measured.

***2.7 Healthy Heart Harvestation*:**

Studies were also performed to determine the activity of SIRT1 and expression of SIRT1, HSP70, PGC-1α, HSF1, eNOS, and iNOS in healthy ICR mice hearts following treatment with SIL.

Experimental Protocol 3: Protein Expression and Activity Assessment 24 h after pre-treatment. (See Figure 4) In these studies, SIL (0.7 mg/kg), RSV (5 mg/kg), or Saline (volume matched 0.2ml) was given *ip* in ICR mice and 24 h later, the mice hearts were harvested for expression and activity assays. The mice were anesthetized with the injection

of pentobarbital sodium (70 mg/kg ip), their hearts were removed, and frozen at -80 degrees Celsius until use.

Experimental Protocol 4. SIRT1 Activity Assessment after 7 days of pre-treatment. In these studies, SIL (0.7 mg/kg), SIL (1.4 mg/kg), RSV (5 mg/kg), or Saline (volume matched 0.2ml) was given *ip* in ICR mice twice a day, once in the morning and once in the evening, for a period of one week. After 7 days, the mice were anesthetized with the injection of pentobarbital sodium (70 mg/kg ip), their hearts were removed, and frozen at -80 degrees Celsius until use.

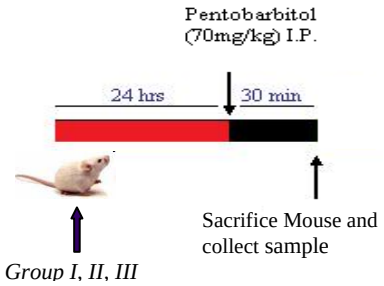


Figure 4. *Experimental Protocol 3: Protein Expression and Activity Assessment 24 h after pre-treatment. ip* injections were given as follows: Group I: Saline (volume matched, 0.2ml); Group II: Resveratrol (5mg/kg, 0.2ml); Group III: Sildenafil (0.7mg/kg, 0.2ml)

2.8 Isolation of Ventricular Cardiomyocytes:

The ventricular cardiomyocytes were isolated using an enzymatic technique similar to a previously reported method.[46] In brief, the mouse was anesthetized with the injection of pentobarbital sodium (70 mg/kg ip), and the heart was quickly removed from the chest. Within 3 min, the aorta was cannulated onto a Langendorff apparatus, and the heart was retrogradely perfused (37 °C) at a constant pressure of 55 mm Hg for 5 min with a Ca^{2+}-free bicarbonate-based buffer containing 120 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5.6 mM glucose, 20 mM $NaHCO_3$, 10 mM 2,3-butanedione monoxime, and 5 mM taurine that was continuously gassed with 95% O_2 + 5% CO_2. The enzymatic digestion was started by adding collagenase type II (Worthington; 0.5 mg/ml each) and protease type XIV (0.02 mg/ml) to the perfusion buffer and continued for 15 min. 50 µM Ca^{2+} was then added in to the enzyme solution for perfusing the heart for another 10–15 min. The digested ventricular tissue was cut into small pieces and gently aspirated with a transfer pipette for facilitating the cell dissociation. The cell pellet was resuspended for a three-step Ca^{2+} restoration procedure (*i.e.* 125, 250, and 500 µM Ca^{2+}). The freshly isolated cardiomyocytes were then suspended in minimal essential medium (catalogue number M1018, pH 7.35–7.45; Sigma) containing 1.2 mM Ca^{2+}, 12 mM $NaHCO_3$, 2.5% fetal bovine serum, and 1% penicillin-streptomycin. The cells were then plated onto 35-mm cell culture dishes that were precoated with 20 µg/ml mouse laminin in phosphate-buffered saline with 1% penicillin-streptomycin for 1 h. After 1 h of plating, the cardiomyocytes were incubated with 1uM SIL, 1uM RSV, or without the drugs (control). The cells were

scraped from the plate following incubation and frozen at -80 degrees Celsius until used for SIRT1 activity analysis and protein expression analysis.

2.9 Western blot analysis for SIRT1, HSP70, HSF1, eNOS, iNOS, and PGC-1α:

Homogenization and Protein Extraction: The murine hearts were homogenized and protein expression was analyzed using techniques similar to a previously reported method.[45] In brief, the hearts were removed from the -80 degree Celsius freezer and a mortar and pestle were used to grind the heart into fine pieces. Total soluble protein was extracted from the myocytes and whole heart lysates with extraction buffer containing RIPA buffer, PMSF, Protease Inhibitor, Na_3VO_4, and β-mercaptoethanol. A mechanical homogenizer was used to further homogenize the samples, then the samples were sonicated and left on ice for 30 minutes. The homogenate was then centrifuged at 14,000 x *g* for 15 min under 4 °C and the supernatant was recovered. The supernatant was frozen at -80 degrees Celsius until used. The protein amount was quantified using a Bio-Rad assay. 100 µg of protein from each sample was separated by SDS-PAGE on a 7.5% polyacrylamide gel, transferred onto nitrocellulose membrane and then blocked with 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.1% Tween 20) for 1 h. The membrane was incubated with primary antibody at a dilution of 1:1000 for each of the respective proteins, i.e. SIRT1, HSP70, HSF1, eNOS, iNOS, PGC-1α, and β-actin overnight at 4 degrees Celsius or at room temperature for 2 h. The membrane was washed with TBST and incubated with the appropriate secondary antibody (1:2000 dilution, 1 h at room temperature). The blots were developed using a chemiluminescent system (ECL Plus;

Amersham Biosciences). Densitometry was used to quantify the optical density for each band and was normalized with the intensity of β-actin.[46]

2.10 Protein purification of SIRT1 for deacetylase activity analysis:

Homogenization and Protein Extraction: The sample preparation techniques used were previously reported.[24] In brief, the hearts were removed from the freezer, and ground with a mortar and pestle in liquid nitrogen. The tissues were homogenized mechanically in a lysis buffer containing 10 mM Tris-HCl (pH 7.4); NP-40 0.5%; 250 mM sucrose; 0.1mM EGTA; 10mM NaCl; 15 mM MgCl2; 1 mM PMSF; 1 mM Na3VO4; and 1 mM NaF. The tissue homogenates (without protease inhibitors) were spun through 4 mL of sucrose 30%, 10 mM Tris HCl (pH 7.5), 10 mM NaCl, and 3 mM $MgCl_2$ at 1,300 x *g* for 10 min at 4°C; the pellet was washed with cold 10 mM Tris-HCl (pH 7.5) and 10 mM NaCl. The nuclei were suspended in 100 uL of extraction buffer containing 50 mM Hepes KOH (pH 7.5), 420 mM NaCl, 0.5 mM EDTA Na_2, 0.1 mM EGTA, and glycerol 10%, sonicated for 30 s, and stood on ice for 30 min. After centrifugation at 13,000 rpm for 10 min, an aliquot of the supernatant (crude extract nuclear) was used to determine protein concentration using a Bio-Rad assay.[24]

Immunoprecipitation. The samples were immunoprecipitated with SIRT1 antibody according to the manufacturers' instructions. 1 μg of SIRT1 primary antibody (Cyclex) was incubated with 250μg of protein in extraction buffer (total volume 200ul) overnight at 4 degrees Celsius. Protein A agarose beads were then incubated with the mixture overnight at 4 degrees C. The mixture was then microcentrifuged for 30 seconds at 4

degrees Celsius. The pellet was washed three times with 250 ul of 1x Cell Lysis Buffer (Cyclex) and 250 ul of Sir2 assay buffer (50 mM Tris-HCl, pH 8.8, 4 mM MgCl2, 0.5 mM DTT). The pellet was then suspended in 100ul of Sir2 assay buffer and kept frozen at -20 degrees Celsius until use.

2.11 SIRT1 deacetylase activity assay:

SIRT1 deacetylase activity was evaluated in the whole heart lysates as well as myocyte lysates from the ICR mice according to the manufacturers' protocols with a few modifications. We measured SIRT1 using a deacetylase fluorometric assay kit (Sir2 Assay Kit, CycLex, Ina, Nagano, Japan). The final reaction mixture (100 *u*L) contained 50 mM Tris-HCl (pH 8.8), 4 mM $MgCl_2$, 0.5 mM DTT, 0.25 mA/mL Lysyl endopeptidase, 1*u*M Trichostatin A, 200 *u*M NAD, and 10 *u*L of crude extract nuclear sample. The fluorescence intensity at 440 nm (exc.340 nm) was measured every 5 minutes for a total of 30 min immediately after the addition of fluorosubstrate peptide, and then once immediately after the addition of stop solution. 60uM of Sirtinol was used as the inhibitor of SIRT1[2432], and Trichostatin A (TSA) was used as an inhibitor of class I and class II HDACS.[28] All determinations were performed in duplicate and the results are reported as relative fluorescence (Arbitrary Units).[24]

2.12 Data analysis and statistics:

Data are presented as mean ± S.E. The differences between groups were analyzed with one-way analysis of variance followed by Student-Newman-Keuls post hoc test for pair-wise comparison. $p < 0.05$ was considered to be statistically significant.[45]

RESULTS

3.1 Exclusions

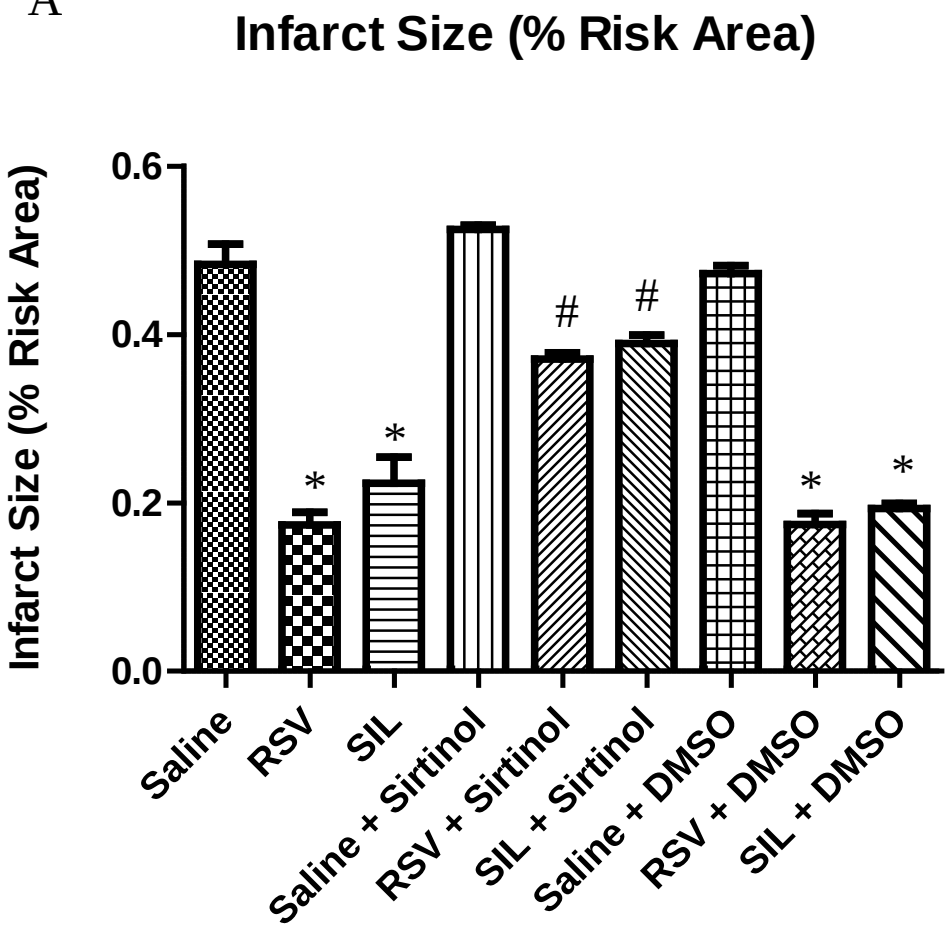
A total of 167 hearts were used in all of the experimental protocols. Among these, 35 hearts were excluded due to one or more of the following reasons: death during any portion of the surgical procedure, damage to the aorta during cannulation, and longer cannulation time (greater than 3 min). Seven mice passed away when Sirtinol was dissolved in 100% DMSO and administered via *ip* injection to the mice. The overall mortality rate was 21%. Mice were randomly assigned to groups in different experimental protocols.

3.2 Sildenafil and Resveratrol preconditioning reduced infarct size

Previous studies have established the cardioprotective effects of SIL and RSV on infarct size following I-R injury.[3, 6, 14-16] Our results confirm previous results as shown in Figure 5. As shown in Figure 5A, the infarct size of the Saline control mice was 48.3 ± 5.9% (n = 6) of the risk area. SIL significantly ($P<0.001$) reduced infarct size to 22.3 ± 7.6% (n = 6) of the risk area in mice, which caused a 53.8% infarct size reduction when administered 24 hours prior to I-R injury compared to the saline controls. RSV, a putative activator of SIRT1, also significantly ($P<0.001$) reduced infarct size to 17.4 ± 3.7% (n = 6)

of the risk area in mice, which was a 64.0% reduction compared to saline controls. Since Sirtinol was dissolved in 10% DMSO, three groups were included in the study to control for the possible effects of DMSO solvent. Infarct size in the Sirtinol vehicle controls (Saline + DMSO, RSV + DMSO, and SIL + DMSO) were not statistically significantly different ($P>0.05$) from the drugs administered alone (See Figure 5A).

A



(n = 6/Group, * = P < 0.001 vs. Saline Controls,
= P < 0.05 vs. other groups)

Figure 5. A. Myocardial Infarct Size. Adult male ICR mice were randomly divided into 9 groups as described in chapter 2.4, Figure 1. Infarct size was significantly (P<0.001 vs. saline controls) reduced in the Sildenafil-treated, Resveratrol-treated, Sildenafil + DMSO-treated, and Resveratrol + DMSO-treated groups. Sirtinol abolished the cardioprotective effects of both Sildenafil and Resveratrol. n = 6/Group, * = P< 0.001 vs. Saline Controls, # = P < 0.05 vs. other groups

B

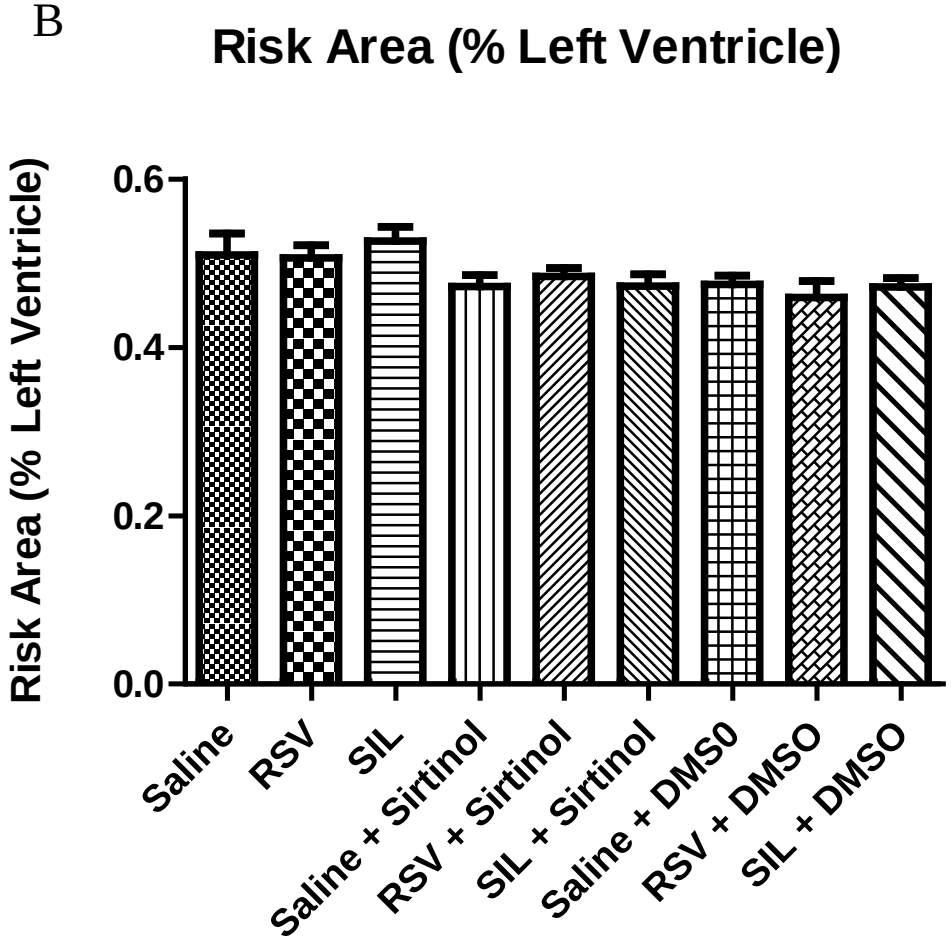


Figure 5B. Myocardial Risk Area. The total risk area was determined to be the percentage of the total left ventricular area that underwent ischemia. n = 6/Group, P>0.05 between all groups.

3.3 Sirtinol blocked the Preconditioning effect of Sildenafil and Resveratrol

Sirtinol is a widely used putative inhibitor of SIRT1.[24] The pharmacological preconditioning effects of Resveratrol are believed to be through the activation of SIRT1. To verify the potential role of SIRT1 in SIL induced cardioprotection, Sirtinol was administered to the mice via interperitoneal injection 30 minutes prior to ischemia. Sirtinol abrogated the cardioprotective effect of 24 h pretreatment with Sildenafil and 24 h pretreatment with Resveratrol as shown in Figure 5A. The infarct sizes of SIL + Sirtinol treated mice (38.9 ± 2.5% of risk area, n = 6) and RSV + Sirtinol treated mice (37.1 ± 1.8% of risk area, n = 6) were not statistically significantly different ($P>0.05$) from mice treated with Saline (48.3 ± 5.9% of risk area, n = 6) or Saline + Sirtinol (52.5 ± 1.2% of risk area, n = 6). The infarct sizes of SIL + Sirtinol treated mice were 74.2% greater than the infarct sizes of SIL treated mice (n = 6). Also, as expected, the infarct sizes of RSV + Sirtinol treated mice were 113.3% greater than the infarct sizes of RSV treated mice (n = 6).

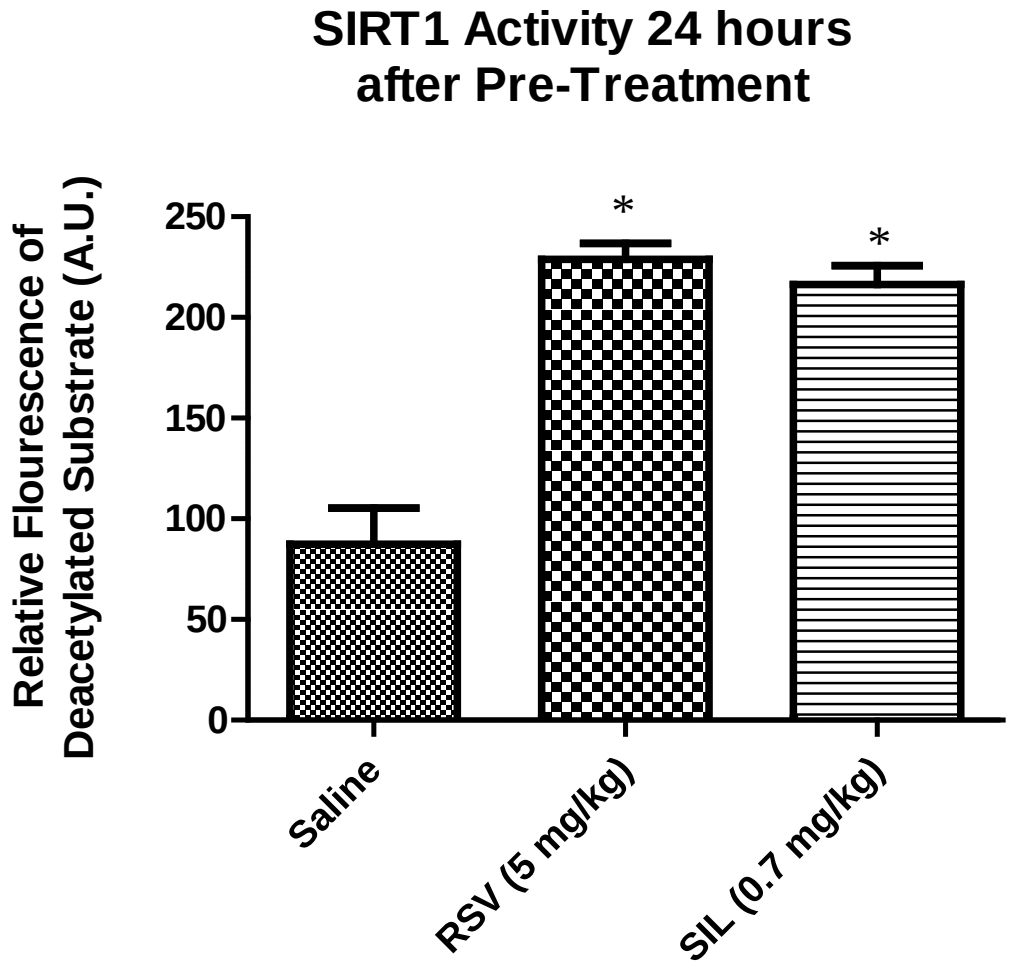
3.4 Sildenafil increased SIRT1 activity in vivo and in vitro

The effect of SIL on SIRT1 activity was investigated by measuring the relative fluorescence of deacetylated SIRT1 substrate (arbitrary units) in homogenized heart samples of ICR mice.

ICR mice treated with Saline, RSV, or SIL 24 h prior to harvesting their hearts were analyzed for SIRT1 activity. As shown in Figure 6, there was a significant increase in SIRT1 activity following SIL and RSV pre-treatment vs. Saline controls ($P<0.001$).

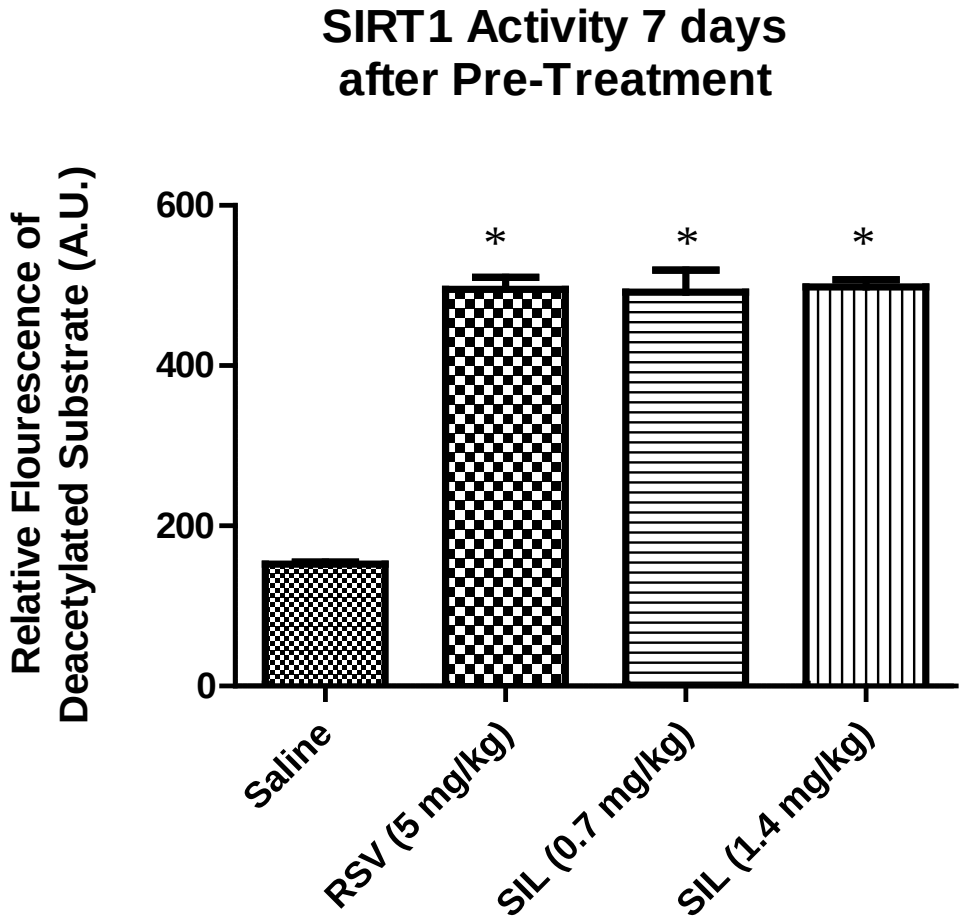
ICR mice were treated twice a day for seven days with Saline, RSV, or SIL (See Materials and Methods: Experimental Protocol 4), then sacrificed to harvest their hearts. As shown in Figure 7, there was a significant increase in SIRT1 activity in the SIL-treated and RSV-treated mice compared to Saline-treated mice. There was not a significant difference ($P>0.05$) of SIRT1 activity between mice given 0.7mg/kg SIL *ip* twice daily vs. 1.4 mg/kg SIL *ip* twice daily administered for 7 days. The difference between SIRT1 activity in SIL-treated and RSV-treated mice was not statistically significant ($P>0.05$).

In another study, ICR mice were pretreated with Saline, RSV, and SIL 24 hours prior to I-R injury, given Sirtinol 30 minutes prior to I-R, and their hearts were harvested after 24 hours of reperfusion (See Figure 2, Experimental Protocol 2). There was a significant difference in the SIRT1 activity observed between RSV-treated I-R mice and SIL-treated I-R mice versus the Saline treated controls ($P<0.0001$). The SIRT1 activities of RSV-treated and SIL-treated mice were also significantly different ($P<0.05$) than the SIRT1 activity of Saline I-R, RSV I-R, and SIL I-R mice (See Figure 8). The direct affect of SIL on SIRT1 activity was also investigated in an in vitro mouse cardiomyocyte model. The adult ICR mice cardiomyocytes were incubated under 37°C and 5% CO_2, for 1 h with 1uM SIL, 1uM RSV, or without any drugs (See Materials and Methods, 2.8 Isolation of Ventricular Cardiomyocytes). The results are shown in Figure 9. The relative fluorescence of deacetylated SIRT1 substrate in myocytes treated with Sildenafil was 450% greater than the control.



(n = 3/Group, * = P < 0.001 vs. Saline)

Figure 6. SIRT1 deacetylase activity in homogenized murine hearts following 24 h of Saline, RSV (5 mg/kg), SIL (0.7 mg/kg), and SIL (1.4 mg/kg) *ip* administration twice a day. n = 3/Group, * = P<0.001 vs. Saline.



(n = 3/Group, * = P < 0.0001 vs. Control)

Figure 7. SIRT1 deacetylase activity in homogenized murine hearts following 7 days of Saline, RSV (5 mg/kg), SIL (0.7 mg/kg), and SIL (1.4 mg/kg) *ip* administration twice a day. n = 3/Group, * = P<0.0001 vs. Control

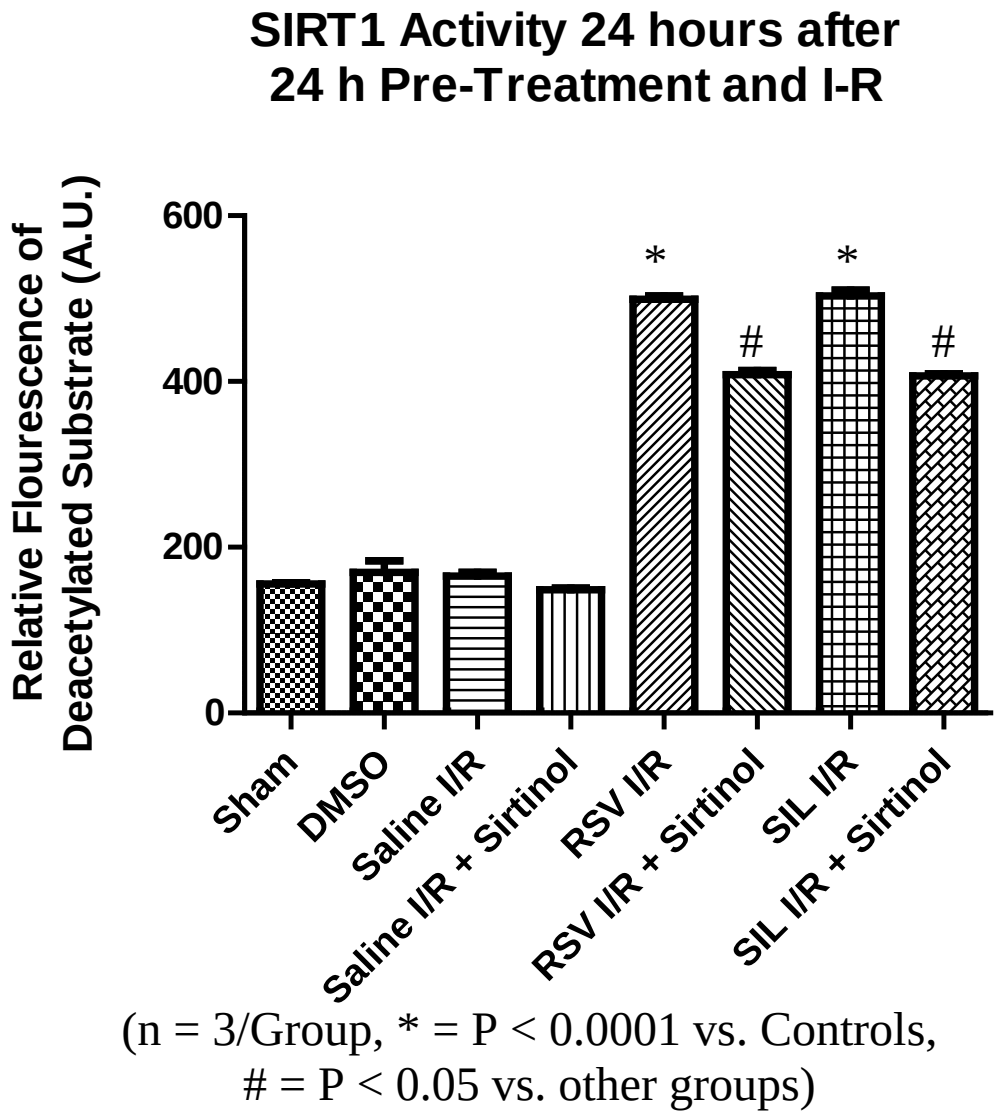
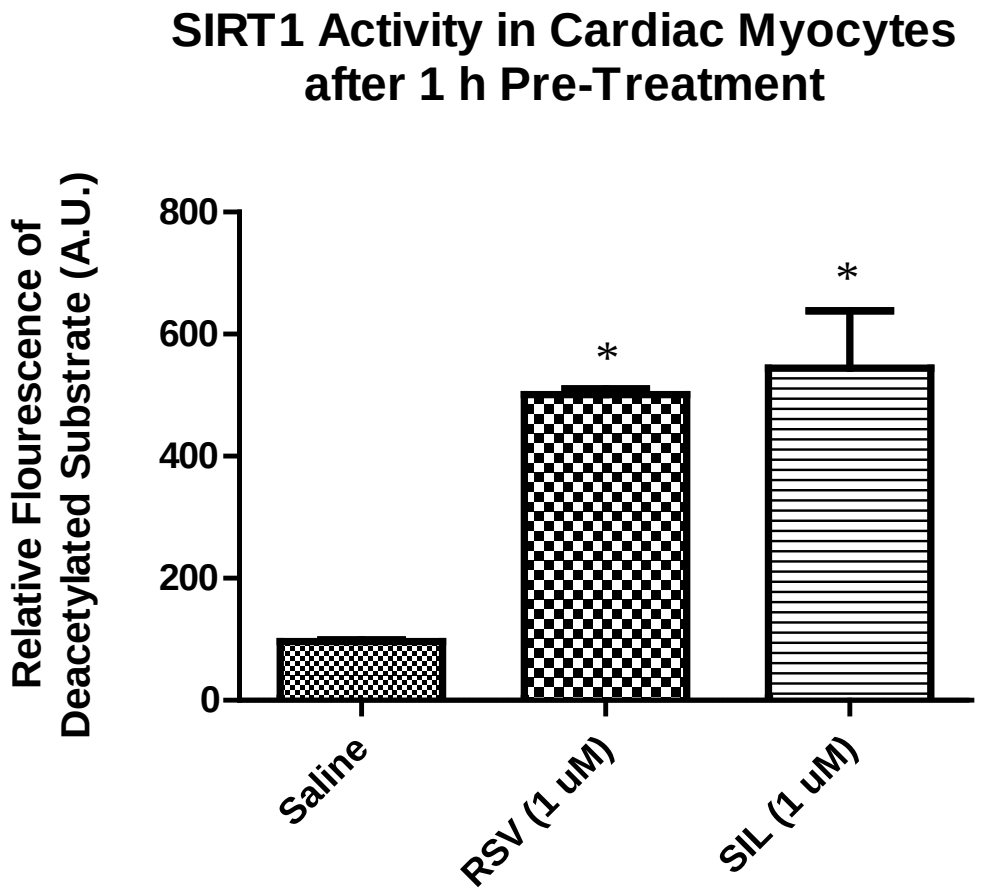


Figure 8. SIRT1 deacetylase activity in homogenized murine hearts pretreated with Saline, RSV (5 mg/kg), and SIL (0.7 mg/kg) 24 hours prior to I-R injury, given Sirtinol (5 mg/kg) 30 minutes prior to I-R, and harvested after 24 hours of reperfusion. Sham mice were used as surgical controls, Saline treated mice were used as drug vehicle controls, and DMSO was used as the Sirtinol vehicle control. n = 3/Group, * = P<0.0001 vs. Controls, # = P < 0.05 vs. other groups.



(n = 2-3/Group, * = P < 0.05 vs. Control)

Figure 9. SIRT1 deacetylase activity in murine cardiac myocytes following 1 h treatment with RSV (1μM), SIL (1μM), or no drug (control). n = 2-3/Group, * P<0.05 vs. Control.

3.5 Cardiovascular function

Doppler echocardiography was used to measure several parameters of left ventricular function in mice treated with drugs according to the experimental Figure 1 at baseline (pre-ischemia) and 24 hours post-reperfusion. These included end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), anterior wall diastolic thickness (AWDT), anterior wall systolic thickness (AWST), posterior wall diastolic thickness (PWDT), posterior wall systolic thickness (PWST), fractional shortening (FS), heart rate (HR), and ejection fraction (EF). All preintervention baseline values were similar and not statistically significantly different ($P>0.05$) for all the sample groups in all of the Doppler echocardiography measurements for cardiovascular function. (See Table 1 and 2)

		LVEDD (mm)	LVESD (mm)	AWDT (mm)	AWST (mm)	PWDT (mm)	PWST (mm)
Sham							
	Baseline	3.80 ± 0.32	2.53 ± 0.25	0.99 ± 0.05	1.37 ± 0.13	0.85 ± 0.17	1.23 ± 0.19
	Post I-R	3.56 ± 0.48	2.56 ± 0.62	0.93 ± 0.13	1.18 ± 0.14	0.89 ± 0.12	1.14 ± 0.30
DMSO							
	Baseline	4.2 ± 0.09	3.18 ± 0.11	0.85 ± 0.08	1.19 ± 0.11	0.89 ± 0.04	1.17 ± 0.07
	Post I-R	4.4 ± 0.28	3.70 ± 0.49	0.89 ± 0.21	1.00 ± 0.35	0.92 ± 0.17	1.11 ± 0.07
Saline							
	Baseline	3.80 ± 0.68	2.55 ± 0.11	0.91 ± 0.8	1.36 ± 0.41	0.84 ± 0.44	1.27 ± 0.61
	Post I-R	3.99 ± 0.77	3.02 ± 0.65	0.95 ± 0.12	1.27 ± 0.08	0.99 ± 0.23	1.57 ± 0.67
RSV							
	Baseline	3.55 ± 0.39	2.46 ± 0.36	0.76 ± 0.25	1.15 ± 0.19	1.07 ± 0.25	1.31 ± 0.24
	Post I-R	3.57 ± 0.40	2.51 ± 0.43	0.90 ± 0.07	1.30 ± 0.16	1.19 ± 0.43	1.48 ± 0.48
SIL							
	Baseline	3.94 ± 0.17	2.26 ± 0.34	0.92 ± 0.30	1.49 ± 0.41	0.9 ± 0.42	1.41 ± 0.39
	Post I-R	3.61 ± 0.16	2.67 ± 0.15	0.80 ± 0.17	0.98 ± 0.14	0.95 ± 0.31	1.29 ± 0.28
Saline + Sirtinol							
	Baseline	3.86 ± 0.38	2.6 ± 0.50	0.81 ± 0.04	1.21 ± 0.16	0.83 ± 0.04	1.17 ± 0.13
	Post I-R	3.66 ± 0.19	2.78 ± 0.26	0.92 ± 0.20	1.20 ± 0.28	1.14 ± 0.11	1.28 ± 0.19
RSV + Sirtinol							
	Baseline	3.84 ± 0.32	2.84 ± 0.35	0.82 ± 0.05	1.16 ± 0.05	0.82 ± 0.16	1.02 ± 0.21
	Post I-R	3.99 ± 0.32	3.38 ± 0.38	0.96 ± 0.22	1.19 ± 0.25	1.01 ± 0.19	1.17 ± 0.27
SIL + Sirtinol							
	Baseline	4.11 ± 0.082	3.06 ± 0.09	0.69 ± 0.11	0.95 ± 0.10	0.73 ± 0.07	1.02 ± 0.03
	Post I-R	3.99 ± 0.30	3.33 ± 0.54	0.73 ± 0.30	0.82 ± 0.42	0.8 ± 0.05	1.01 ± 0.15

Table 1. Cardiovascular Function: Doppler Echocardiography results. Left ventricular contractile function: left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), anterior wall diastolic thickness (AWDT), anterior wall systolic thickness (AWST), posterior wall diastolic thickness (PWDT) and posterior wall systolic thickness (PWST).

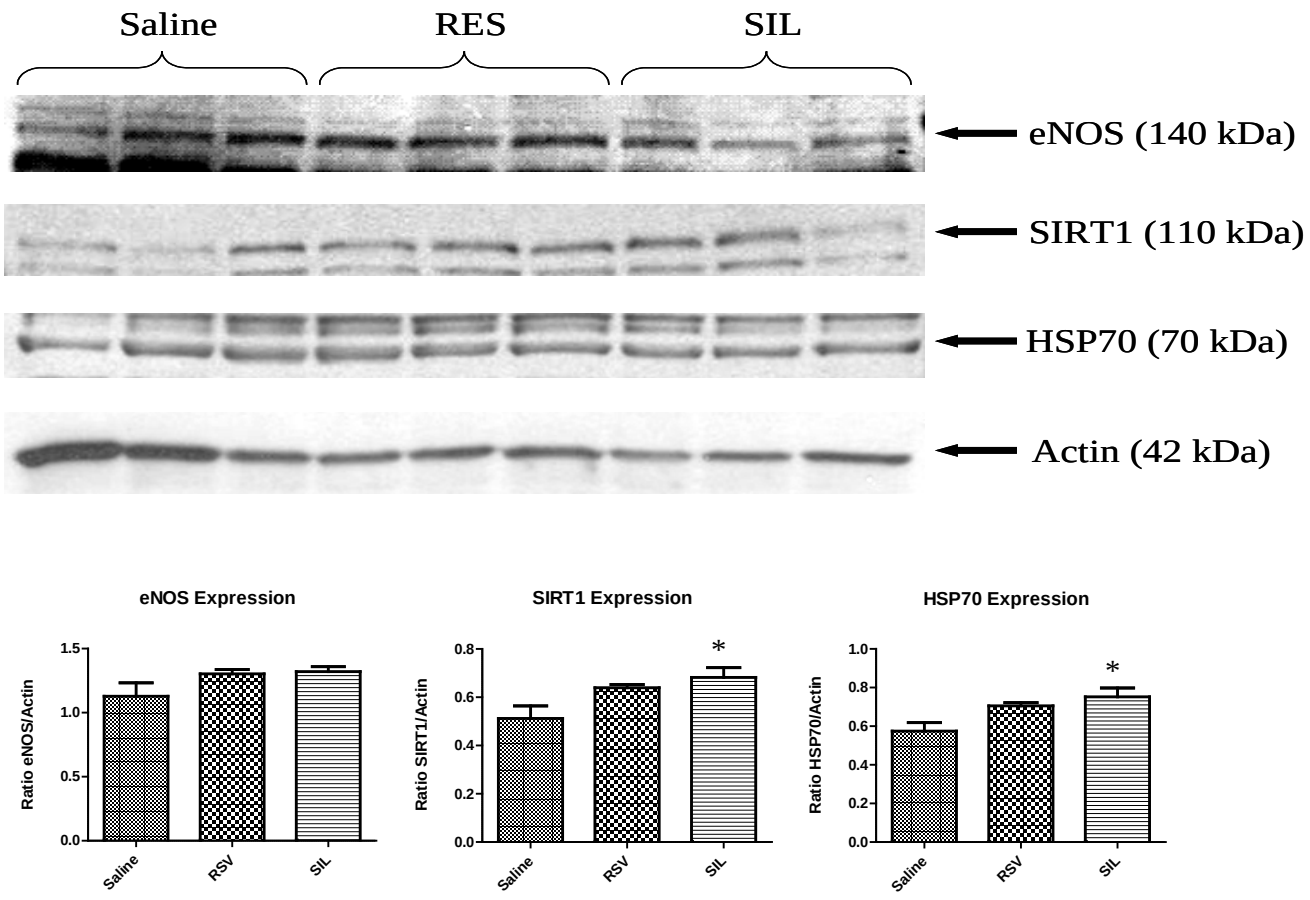
		FS (%)	EF (%)	HR (BPM)
Sham				
	Baseline	37.57 ± 2.95	67.92 ± 3.80	309 ± 74.6
	Post I-R	33.74 ± 7.49	62.43 ± 9.33	425 ± 56.3
DMSO				
	Baseline	28.54 ± 4.95	54.88 ± 7.69	326 ± 85.9
	Post I-R	17.04 ± 8.1	34.98 ± 14.34	344 ± 4.35
Saline				
	Baseline	35.42 ± 11.84	64.25 ± 15.61	267 ± 17.8
	Post I-R	24.51 ± 3.25	49.07 ± 5.35	313 ± 81.0
RSV				
	Baseline	26.99 ±9.31	52.94 ± 15.47	340 ± 64.7
	Post I-R	29.97 ± 7.24	57.61 ± 11.1	434 ± 63.5
SIL				
	Baseline	42.87 ± 9.96	73.65 ± 10.28	297 ± 36.1
	Post I-R	25.95 ± 2.63	51.82 ± 4.24	361 ± 77.0
Saline + Sirtinol				
	Baseline	36.68 ± 7.79	66.40 ± 10.79	258 ± 43.9
	Post I-R	31.17 ± 7.39	59.13 ± 10.41	384 ± 49.5
RSV + Sirtinol				
	Baseline	32.94 ± 2.41	62.02 ± 3.70	388 ± 48.3
	Post I-R	19.01 ± 2.74	39.54 ± 5.09	415 ± 27.2
SIL + Sirtinol				
	Baseline	29.77 ± 2.20	57.13 ± 3.30	222 ± 7.57
	Post I-R	19.13 ± 7.30	39.21 ± 13.03	334 ± 23.1

Table 2. Cardiovascular Function: Doppler Echocardiography results. Left ventricular contractile function: fractional shortening (FS), heart rate (HR), and ejection fraction (EF).

3.6 Western Blot analysis of SIRT1, eNOS, iNOS, HSF1, HSP70, and PGC-1α

To investigate the effect of SIL on the expression of signaling molecules involved in the upregulation of SIRT1, western blot analysis was performed as described in materials and methods: 2.10 Western blot analysis for SIRT1, HSP70, HSF1, eNOS, iNOS, and PGC-1α. The results shown in Figure 10 show a significant increase in SIRT1 expression, eNOS expression, and HSP70 expression ($P<0.05$) in homogenized adult ICR mice treated with 0.7 mg of SIL 24 hours prior to harvesting the mouse heart as described in materials and methods: 2.7 Healthy Heart Harvestation, Experimental Protocol 3. Further studies were performed to investigate the effect of SIL on the expression of iNOS, PGC-1α, and HSP70 following I-R injury as described in materials and methods: 2.4 Myocardial Infarction Protocol, Experimental Protocol 2 (See Figure 2). The results are shown in Figure 11 show a significant increase in iNOS expression and PGC-1α expression ($P<0.05$) compared to the Saline controls. HSP70 expression following the administration of SIL and I-R injury also showed an increase in HSP70 expression however the results were not statistically significant ($P>0.05$).

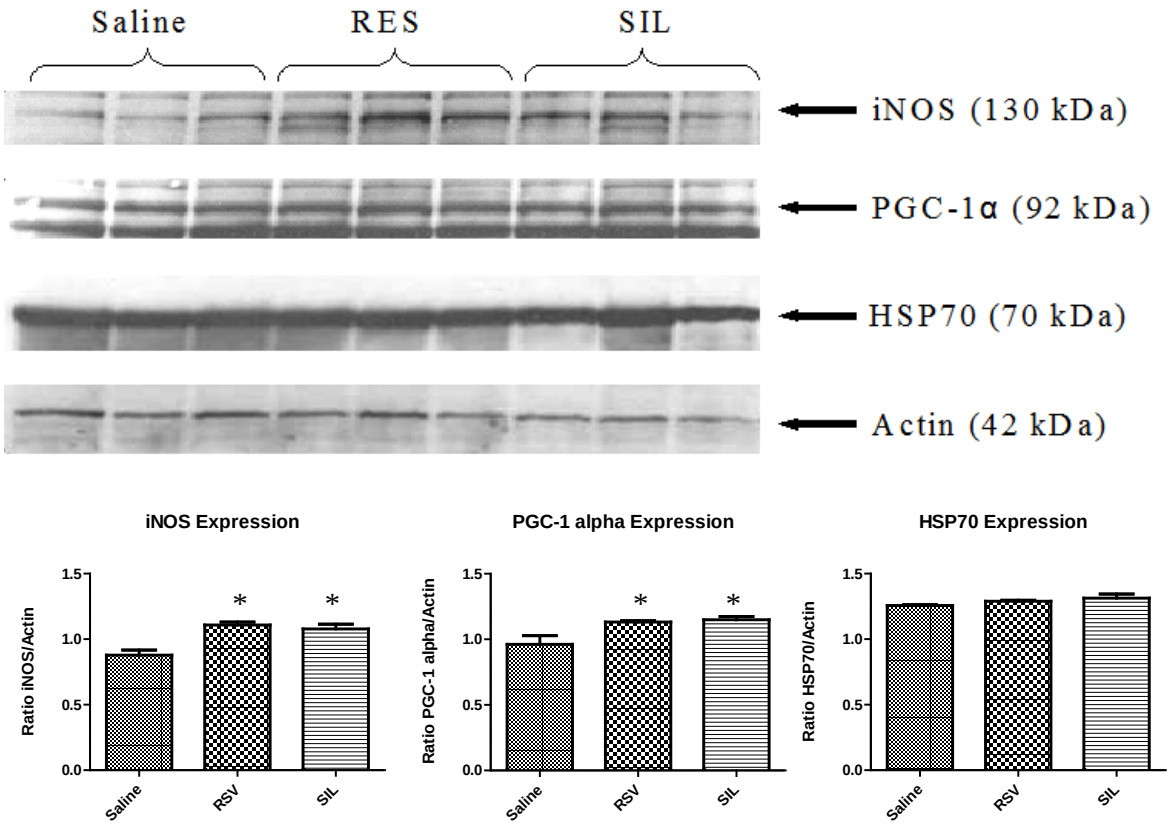
24 hour Pre-Treatment



(n = 3/Group, * = P < 0.05 vs. Saline)

Figure 10. Cardiac expression of eNOS, SIRT1, and HSP70 in mice administered SIL (0.7 mg/kg), RSV (5 mg/kg), or Saline (volume matched 0.2ml) *ip* and sacrificed 24 h later for heart harvestation without I-R. *Top:* Representative western blots showing eNOS, SIRT1, and HSP70 protein expression. *Bottom:* Densitometric quantification of western blots normalized against the actin level for each sample. n = 3/Group, * = P<0.05 vs. Saline.

24 hour pre-treatment, 30 minutes Ischemia, 24 hours Reperfusion



(n = 3/Group, * = P < 0.05 vs. Saline)

Figure 11. Cardiac expression of iNOS, PGC-1α, and HSP70 in mice treated with SIL (0.7 mg/kg), RSV (5 mg/kg), or Saline (volume matched 0.2ml) *ip* and 24 h later were subjected to 30 minutes of ischemia and 24 h of reperfusion before heart harvestation. *Top:* Representative western blots showing iNOS, PGC-1α, and HSP70 protein expression. *Bottom:* Densitometric quantification of western blots normalized against the actin level for each sample. n = 3/Group, * = P<0.05 vs. Saline

DISCUSSION

4.1 Rationale of the ***Present study***

The concept of using Sildenafil as a pharmacological preconditioning agent has been shown previously in our lab.[6, 16] Resveratrol, a putative activator of SIRT1 has also been demonstrated to be a pharmacological preconditioning agent.[14-16, 25] The cellular and molecular mechanisms mediating the cardioprotective mechanisms of SIRT1 have not been elucidated, and the aim of this study was to determine if SIRT1 acts as a mediator in the cardioprotective pathway of SIL following I-R injury. SIRT1 has been shown to modulate common factors involved in the cardioprotective pathway of SIL, such as eNOS, iNOS, HSP70, and HSF1.[14, 43, 47] Based on these studies, we postulated that SIL treatment *ip* would increase SIRT1 activity and expression. To test this hypothesis a murine model was used in which healthy hearts, hearts following I-R injury, and cardiac myocytes were analyzed. The effects of SIL were compared to the positive control RSV, the negative control Saline, and administration with Sirtinol, an inhibitor of SIRT1. It was also hypothesized that SIL treatment would increase common factors involved in the cardioprotective pathway of SIRT1, and this hypothesis was tested using western blot analysis. Another goal of this study was to determine if SIRT1 had an effect on the cardioprotective effect of SIL following I-R injury. This hypothesis was tested by

analyzing infarct size in pre-treated murine heart samples following I-R and comparing baseline and post I-R function with Doppler echocardiography.

4.2 SIRT1 is a key factor involved in the cardioprotective mechanism of Sildenafil following I-R injury

In the present study, Sirtinol blocked the infarct-limiting effects of SIL when administered to mice 30 minutes prior to ischemia. Since Sirtinol is a putative inhibitor of SIRT1[24], this suggests that SIRT1 is involved in mediating SIL-induced cardioprotective signaling following I-R injury.

4.3 Sildenafil increases SIRT1 activity in vivo and in vitro

The direct effect of SIL on SIRT1 was investigated by measuring SIRT1 activity *in vivo* and *in vitro.* In *in vivo* studies, SIRT1 activity was increased in healthy ICR mice heart homogenates, as well as ICR mice hearts that had undergone I-R injury. This indicates that SIL is either an upstream activator or direct activator of SIRT1. To further investigate the effect of SIL on SIRT1 activity, *in vitro* studies were performed in adult ICR mice myocytes, where 1μM SIL was applied directly on the plated myocytes and incubated under 37 °C and 5% CO_2, for 1 h. As shown in Figure 9, SIRT1 activity was increased 450% compared to the control. This also suggests that SIL is an activator of SIRT1.

4.4 Cardiac Function Studies

In the previous studies, our laboratory has shown that Sildenafil improves cardiac function following I-R injury. We have shown significantly less LVEDD and LVESD, improved FS, and less AWST and AWDT in SIL-treated mice vs. Saline controls after permanent occlusion of coronary artery.[3] We contemplated that the Sirtinol would abrogate the cardioprotective effects of SIL by worsening of cardiac function following I-R injury. However, the results from our studies were inconclusive because we assessed cardiac function 24h after reperfusion in limited number of mice.

4.5 Sildenafil increased expression of common factors modulated by SIRT1

To further investigate the cardioprotective pathway of SIL, we assessed the expression of SIRT1, eNOS, iNOS, HSF1, HSP70, and PGC-1α following treatment with SIL. Our previous studies have established that SIL induces cardioprotective effects through NO generated from eNOS and iNOS and the opening of mitoK_{ATP} channels.[6, 9, 18] Moreover, we have demonstrated a significant increase in eNOS and iNOS proteins 24 h post-MI after treatment with SIL vs. Saline.[3] In the present study also, we observed an increase in eNOS and iNOS proteins 24 hours post I-R injury after treatment with SIL. SIRT1 has also been shown to upregulate eNOS expression and increase endothelium relaxation.[47] NO has a role in preserving cardiomyocytes through opening mitoK_{ATP} channels, preventing Ca^{2+} overload[3], and attenuating the formation of ROS.[10] ROS are involved in many cardiovascular diseases such as I-R injury, congestive heart failure, and coronary heart disease.[23, 24] Mitochondria are the main energy sources of cells, and

compromised mitochondrial function can lead to cardiovascular and metabolic diseases.[25] PGC-1α controls mitochondrial biogenesis and function, it plays an important role in cell survival during I-R. RSV is believed to exert pharmacological preconditioning by activating PGC-1α through diverse upstream signaling pathways involving SIRT1 and NO.[14] PGC-1α has been shown to be required for the adaptation to the stress-induced change in mitochondrial membrane potential.[34] In this study, we observed an increase in PGC-1α expression in SIL-treated mice suggesting its potential role in preservation of mitochondrial membrane potential as demonstrated previously from our laboratory.[46]

Heat Shock proteins, such as HSP70, play an important role in maintaining cellular homeostasis and aid in cardioprotection against I-R injury. HSF1 is a gene that encodes a transcription factor that regulates gene expression in response to cellular stress, including the human HSP70 gene. Both HSP70 and HSF1 have been shown to be upregulated 24 h following ischemic preconditioning.[13] SIRT1 has been shown to regulate several transcription factors including HSF1 involved in the cellular stress response. Activation of SIRT1 prolongs HSF1 binding to the heat shock promoter of HSP70 which may ensure sustain synthesis of this protein.[43] In the present study, an increase in HSP70 expression in the heart was observed 24 hours in the post-ischemic and non-ischemic hearts after SIL administration.

4.6 Study Limitations

SIRT1 deficient mice were not used in this study because they rarely survive postnatally and usually exhibit heart abnormalities.[35] We realize the potential non-specific

effects of SIRT1 inhibitor, sirtinol. Due to time constraints, transgenic mice overexpressing SIRT1 in heart muscle or siRNA studies could not be conducted. We also could not consider the potential role of other Sirtuins such as SIRT2 in the cytosol and SIRT3 in the mitochondria that might be expressed in the myocytes and may possibly be involved in the cardioprotective pathway of SIL.

4.7 Future Directions

More thorough investigations should be conducted to elucidate the role of SIRT1 in SIL induced cardioprotection against I-R injury. The 24 h time frame of reperfusion was selected to mimic the second window of ischemic preconditioning, allowing for transcriptional changes to occur and to effect protein expression. Future studies can be done at different reperfusion time-points to determine the mechanism of cardioprotection of SIL through the activation of SIRT1. The regulatory mechanisms that control SIRT1 activity need to be elucidated for the appropriate use of pharmacological agents such as SIL in treatment of various disease processes. Some studies have shown that the extent of SIRT1 expression also affects disease processes. Lower levels of SIRT1 expression have shown protective mechanisms, and greater levels of SIRT1 expression have contributed to the progression of disease processes.[35, 36] The therapeutic potential of SIRT1 needs to be carefully evaluated at various dosages.

4.6 Clinical implications

SIRT1 has been shown to protect against cardiovascular diseases, cancer, and neurodysfunctions.[32] Pharmacological interventions to regulate SIRT1 activity have been successful in the past, and it is conceivable that in the near future more selective and powerful drugs can be used for therapeutic interventions in human disorders.[32] In the present study, we have demonstrated that SIL exerts pharmalogical preconditioning by activating SIRT1 and increasing SIRT1 expression, and indicates that SIL may have additional therapeutic benefits through the activation of SIRT1.

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