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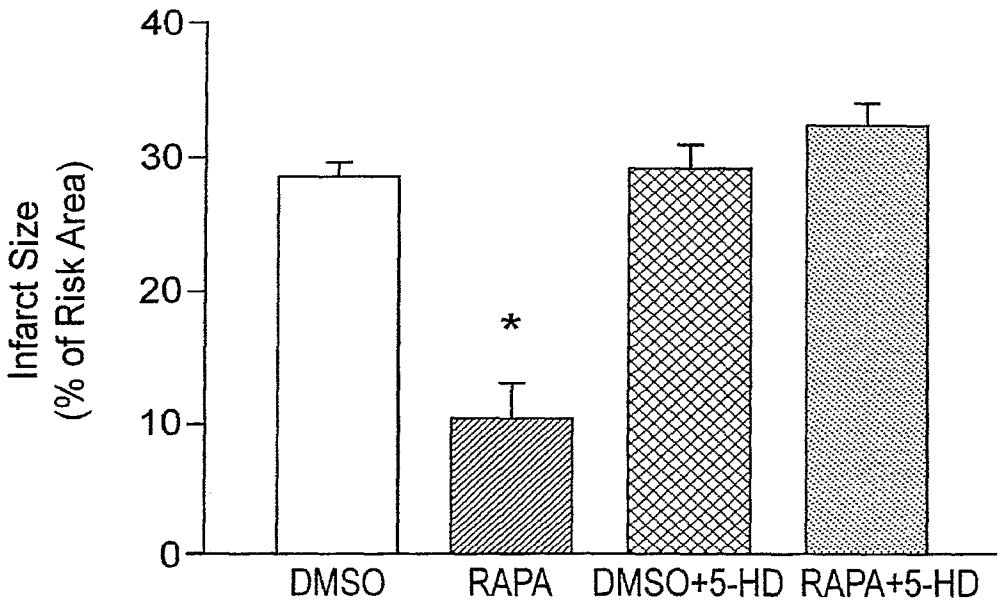
(54) **Title:** CARDIOPROTECTIVE AND OTHER USES OF MAMMALIAN TARGET OF RAPAMYCIN (M-TOR) INHIBITORS



(57) **Abstract:** Novel uses of rapamycin include, e.g., the preconditioning-like effect of rapamycin against infarction in the intact heart as well against necrosis and apoptosis in cardiomyocytes. Other uses, especially in post-heart attack patients and other patients at risk of ischemia/reperfusion injury, are disclosed for rapamycin and other m-TOR inhibitors.

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Cardioprotective and other uses of mammalian target of rapamycin (m-TOR) inhibitors

DESCRIPTION

Field of the Invention

The invention generally relates to medicine, especially cardiac medicine, and especially relates to medicine relating to ischemia/reperfusion injury particularly in the heart but also elsewhere.

BACKGROUND OF THE INVENTION

Since the initial findings by Murry et al. (Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; 74(5):1124-1136) that a brief episode of ischemia can paradoxically protect the heart from subsequent ischemic injury, many investigators have tried to discover a pharmacological solution to ischemic preconditioning (IPC). IPC works by triggering several endogenous protective mechanisms and is realized in two phases: the early phase, which lasts up to 2-3 hours, and a late phase, which appears 12-24 hours later with duration up to 3-4 days. Considerable progress has been made toward identifying cellular triggers and signal transduction mechanisms involved in the process of preconditioning and many pharmacological agents have been shown to induce a preconditioning like-effect (Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev* 2003; 83(4):1113-1151; Kukreja RC, Salloum F, Das A, Ockaili R, Yin C, Bremer YA *et al.* Pharmacological preconditioning with sildenafil: Basic mechanisms and clinical implications. *Vascul Pharmacol* 2005; 42(5-6):219-232.) However, despite significant advances in this field, no agent has yet gained widespread clinical use (Bolli R, Becker L, Gross G, Mentzer R, Jr., Balshaw D, Lathrop DA. Myocardial protection at a crossroads: the need for translation into clinical therapy. *Circ Res* 2004; 95(2):125-134.)

The following patent literature is made of record:

U.S. patent no. 5,496,832 issued Mar. 5, 1996 to Armstrong (American Home Products Corp.) for "Method of treating cardiac inflammatory disease."

U.S. patent no. 6,239,124 issued May 29, 2001 to Zenke et al. (Novartis AG),

for "Pharmaceutical compositions for the treatment of transplant rejection or autoimmune or inflammatory conditions comprising cyclosporine A and 40-O-(2-hydroxyethyl)-rapamycin."

U.S. patent no. 6,670,355 issued Dec. 30, 2003 to Azrolan et al. (Wyeth), titled "Method of treating cardiovascular disease."

U.S. patent publication no. 2004/0097538 published May 20, 2004 by Azrolan et al., for "Method of treating cardiovascular disease."

U.S. patent publication no. 20050209266 published Sept. 22, 2005 by Garvey for "Nitrosated and nitrosylated rapamycin compounds, compositions and methods of use."

U.S. patent no. 7,060,709 issued June 13, 2006, to Cooperstone et al. (Wyeth), titled "Method of treating hepatic fibrosis."

U.S. patent publication no. 20060135549 published June 22, 2006 by Graziani et al. (assigned to Wyeth), titled "Rapamycin analogues and the uses thereof in the treatment of neurological, proliferative, and inflammatory disorders."

U.S. patent no. 7,091,207 issued Aug. 15, 2006, to Rakesh Kukreja, titled "Method of treating myocardial infarction with PDE-5 inhibitors".

U.S. patent publication no. 20060204537 published Sept. 14, 2006 by Ratner et al., titled "Silicone blends and composites for drug delivery."

U.S. patent publication no. 20060211752 published Sept. 21, 2006 by Kohn et al., titled "Use of phenylmethimazoles, methimazole derivatives, and tautomeric cyclic thiones for the treatment of autoimmune/ inflammatory diseases associated with toll-like receptor overexpression."

U.S. patent publication no. 20060257403 published Nov. 16, 2006 by Young et al., titled "Methods for treating and preventing fibrosis."

U.S. patent publication no. 20060276390 published Dec. 7, 2006 by Aharoni et al., titled "Combined treatments comprising synthetic peptide copolymers for preventing graft rejection."

U.S. patent publication no. 20060280738 published Dec. 14, 2006 by Tedder, titled "Anti-CD19 antibody therapy for transplantation."

U.S. patent publication no. 20060286141 published Dec. 21, 2006 by Campbell, titled "Systems for gel-based medical implants."

U.S. patent no. 7,160,867 issued Jan. 9, 2007, to Abel et al. (Isotechnika, Inc.), for "Rapamycin carbohydrate derivatives."

U.S. patent publication no. 20070014757 published Jan. 18, 2007 by Chauhan et al., titled "Compositions and complexes containing a macromolecular compound as potential anti-inflammatory agents."

U.S. patent publication no. 20070027184 published Feb. 1, 2007 by Malecha, et al., titled "Multicyclic sulfonamide compounds as inhbitors of histone deacetylase for the treatment of disease."

U.S. patent publication no. 20070036768 published Feb. 15, 2007 by Fraser et al., titled "Systems and methods for treating patients with processed lipoaspirate cells."

U.S. patent publication no. 20070071675 published Mar. 29, 2007 by Wu et al., titled "Dual variable domain immunoglobulin and uses thereof."

SUMMARY OF THE INVENTION

Before this invention, there have been no clinically proven drugs that are being used to precondition or protect the heart following ischemia/reperfusion injury. The present invention addresses this and other voids.

In one preferred embodiment, the invention provides a method of protecting against necrosis and/or apoptosis in cardiomyocytes, comprising: administering (such as, e.g., administering via an intraperitoneal administration route, etc.) to a patient having a population of cardiomyocytes an effective dose of a protective substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor.

The invention in another preferred embodiment provides a method of preconditioning a patient against myocardial infarction, comprising: opening mitochondrial KATP channels in the patient by administering (such as, e.g., administering via an intraperitoneal administration route, etc.) to the patient an effective amount of a substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor), such as, e.g., an inventive method of preconditioning a patient against myocardial infarction wherein the substance is administered to the patient following ischemia/reperfusion injury in the heart (such as, e.g., an ischemia/reperfusion injury resulting from coronary bypass surgery and/or angioplasty in the heart); etc.

In another preferred embodiment, the invention provides a cardio-protective method in a post-heart attack patient, comprising: after the patient has experienced a heart attack of a heart muscle of the patient, administering (such as, e.g.,

administering via an intraperitoneal administration route, etc.) to the patient an effective amount (such as, e.g., an amount sufficient to protect against ischemia/reperfusion injury (such as, e.g., ischemia/reperfusion injury that results from coronary bypass surgery and/or angioplasty in the heart) of a cardio-protective substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor; such as, e.g., inventive cardio-protective methods wherein, after the administering step, the heart muscle undergoes ischemia and/or reperfusion having a less-damaging effect to the heart muscle than if the cardio-protective substance had not been administered; inventive cardio-protective methods comprising a step of limiting infarct effect on the heart muscle, provided that the infarct limiting step comprises a step other than improving ventricular function; etc.

The invention in a further preferred embodiment provides a protective method in a post-heart attack patient, comprising: after the patient has experienced a heart attack, administering to the patient an effective amount of a protective substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor, and thereby protecting a tissue selected from the group consisting of: heart, brain, liver, kidney, lung, gut, skeletal muscle, pancreas, retina and intestinal tissue wherein the protecting comprises reducing an effect to the tissue from ischemia/reperfusion injury.

In another preferred embodiment the invention provides a protective method in an at-risk patient (such as, e.g., a post-heart attack patient; a patient who has suffered head trauma; a patient at risk of cardiovascular disease and a patient who has suffered stroke; etc.), comprising: administering to the patient an effective amount of a protective substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor, and thereby protecting a tissue selected from the group consisting of: heart, brain, liver, kidney, lung, gut, skeletal muscle, pancreas, retina and intestinal tissue wherein the protecting comprises reducing an effect to the tissue from ischemia/reperfusion injury.

The invention also provides another preferred embodiment which is a protective method in a post-heart attack patient, comprising: administering to a patient who has experienced a heart attack an amount of a substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target

of rapamycin (mTOR) inhibitor, wherein the amount is an amount effective to prevent a process of heart remodeling.

Further another preferred embodiment of the invention provides a method of screening whether to administer to a patient a substance selected from the group consisting of rapamycin, everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor, comprising: determining whether the patient has a factor selecting from the group consisting of having had a heart attack, having suffered head trauma, being at risk of cardiovascular disease, having suffered stroke, having heart tissue at risk of ischemic/reperfusion injury, having brain tissue at risk of ischemia/reperfusion injury, having liver tissue at risk of ischemic/reperfusion injury, having kidney tissue at risk of ischemic/reperfusion injury, having lung tissue at risk of ischemic/reperfusion injury, having gut tissue at risk of ischemic/reperfusion injury, having skeletal muscle at risk of ischemic/reperfusion injury, having spleen tissue at risk of ischemic/reperfusion injury, having pancreatic tissue at risk of ischemic/reperfusion injury, having retinal tissue at risk of ischemic/reperfusion injury and having intestinal tissue at risk of ischemic/reperfusion injury; preferably followed by a step, if the patient is determined to have at least one such factor, of administering the substance to the patient following the determination that the patient has had at least one such factor.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, aspects and advantages will be better understood from the following detailed description of the preferred embodiments of the invention with reference to the drawings, which are as follows:

Fig. 1. Experimental protocol for isolated heart studies.

Fig. 2. Myocardial infarct sizes. Acute preconditioning with rapamycin (RAPA) reduced infarct size. Infusion of 5-HD during stabilization (RAPA+5-HD) blocked the protective effect of rapamycin in the preconditioned mice (*$P<0.001$ vs. DMSO, DMSO+5-HD, RAPA+5-HD). N = 6 mice/group.

Fig. 3. Cardiac function. (A) Rate-Force Product: There was no significant difference in the recovery of cardiac function (rate-force product) among groups. (B) Coronary flow: There was no significant difference in the change in coronary flow from baseline to reperfusion among the groups. N = 6 mice/group.

Fig. 4. Representative images of adult mouse cardiomyocytes showing effect of rapamycin on myocyte viability. (A) Normal isolated cardiac myocytes; (B)

Myocytes subjected to 40 min of simulated ischemia (SI) and 1 h of reoxygenation (RO). Cell necrosis is evident by the increased number of tryptan-positive blue myocytes. (C) Pretreatment with 100 nM rapamycin reduces the number of tryptan-blue positive myocytes demonstrating improved cell viability.

Fig. 5. Effect of rapamycin on cardiomyocyte viability: 40 min of simulated ischemia (SI) followed by 1 h of reoxygenation (RO) considerably increased the number of tryptan blue positive cells, demonstrating worsening cardiomyocyte necrosis (*P<0.001 vs. control). Pretreatment with rapamycin (25, 50, or 100 nM) for 1 hr prior to simulated ischemia decreased the number of tryptan blue positive cells, indicating improved cell viability with rapamycin (†P<0.001 vs. SI-RO).

Fig. 6. Effect of rapamycin on inhibition of apoptosis in cardiomyocytes. Cells were treated with rapamycin (25, 50, or 100 nM) for 1 h followed by 40 min of simulated ischemia (SI) and 18 h of reoxygenation (RO). Apoptotic nuclei were observed using the TUNEL assay. Apoptotic cell death was markedly increased after SI-RO (*P<0.001 vs. control). Rapamycin pretreatment reduced TUNEL-positive nuclei compared to SI-RO alone (†P<0.001) demonstrating less apoptotic cell death.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

In summarizing inventive embodiments above, there have been mentioned inventive uses of rapamycin, rapamycin being a known composition. "Rapamycin" herein means the chemical nucleus commonly known by that name and includes a compound comprising a rapamycin chemical nucleus. The rapamycin chemical nucleus has chemical formula $C_{51}H_{79}NO_{13}$. Rapamycin is sometimes called "Sirolimus." Rapamycin is commercially available.

The novel uses disclosed herein for rapamycin may be extended to other compounds related to rapamycin such as, e.g., rapamycin analogues. Examples of rapamycin analogues are, e.g., compounds called rapamycin analogues in the literature (such as, e.g., everlimus); compounds derived from rapamycin; compounds having substantial structure similarity to rapamycin; etc.

"Ischemia/reperfusion injury" means the injury inflicted as a result of restoring oxygenated blood in the tissue following a period of no-blood flow. Examples of ischemia/reperfusion injury are, e.g., heart attack, stroke, etc.

"Mammalian target of rapamycin (mTOR) is an enzyme serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival,

protein synthesis, and transcription. An example of an mTOR inhibitor is rapamcyin.

"Opening mitochondrial KATP channels" means the opening of pores in the mitochondrial membrane that facilitate movement of potassium ion under the control of ATP, which is essential for performing cellular functions.

"Protecting" herein means that the extent or magnitude of an undesirable effect (such as, e.g., undesirable necrosis; undesirable apoptosis; undesirable ischemia/ reperfusion injury) is reduced.

"Preconditioning a patient against myocardial infarction" means protecting the heart tissue from dying as a result of deprivation of oxygen or ischemia.

The invention may be further appreciated with reference to the following Examples, without the invention being limited thereto.

EXAMPLE 1

Rapamycin has been shown to protect the heart muscle after experimentally induced heart attack in mice. Rapamycin is now considered an antibiotic that inhibits protein synthesis through mammalian target of rapamycin (mTOR) signaling. This inventive example concerns the role of the drug rapamycin and other mTOR inhibitors (including analogous compounds everolimus) in protection against ischemia/reperfusion (I/R) injury.

In the experiments of this example, adult male ICR mice were treated with rapamycin (0.25 mg/kg, IP) or volume-matched MDSO (solvent for rapamycin). The hearts were subjected to 20 min of global ischemia and 30 min of reperfusion in Langendorff mode. The blocker of mitochondrial K_{ATP} channel, 5-hydroxydecanoate (5-HD, 100 μM) was given 10 min before ischemia. Infarct size in the DMSO treated group was 28.2±1.3% and was reduced to 10.1±2.8% in the rapamycin-treated mice (64% decrease, $P<0.001$). 5-HD blocked the protective effect (infarct area 32.2±1.8%, $P<0.001$ vs. rapamycin). The infarct limiting effect of rapamycin was not associated with improved recovery of ventricular function. This is because the post-ischemic functional improvement in the heart occurs several hours later and the isolated perfused heart model used in these studies does not allow measurement of function during extended period of reperfusions following ischemia.

In this experimental example, also there was examined the effect of rapamycin against necrosis and apoptosis in adult cardiomyocytes subjected to simulated ischemia and reoxygenation. Mycocytes treated with rapamycin in doses from 25-100

nM demonstrated significantly lower tryptan blue positive necrotic cells and TUNEL-positive apoptotic nuclei, supporting the protective role of rapamycin in the intact heart.

These data of this example suggest that rapamycin induces potent preconditioning-like effect against myocardial infarction through opening of mitochondrial K_{ATP} channels. Therefore rapamycin may be used as a therapeutic strategy to limit infarction, apoptosis and remodeling following I/R injury in the heart.

In this example, mTOR inhibitor rapamycin inventively has been used for reducing myocardial infarct size following experimentally-induced heart attack (ischemia) in mice. Also, in this example, it has been shown that rapamycin reduces cardiomyocyte apoptosis (programmed cell death) following simulated ischemia/ reoxygenation.

Therefore, mTOR inhibitors may be used to treat ischemia/reperfusion injury resulting from procedures including coronary bypass surgery and angioplasty in the heart. In addition, mTOR inhibitors can be used to avoid or ameliorate the adverse effects of myocardial infraction including cardiac hypertrophy and heart failure. Also, the mTOR inhibitors can be used to protect ischemia-related injury in other organs including brain, heart, liver, intestine, kidney, lung, gut, spleen, pancreas, nerves, spinal cord, retinal tissue, vasculature, and skeletal muscle.

Rapamycin has now been shown to have a preconditioning-like protective effect in the mouse heart. More specifically, in this example it has been shown that intraperitoneal administration of rapamycin induces powerful cardioprotective effect as demonstrated by significant reduction in infarct size. Furthermore, the findings have been replicated at the cellular level by showing that rapamycin directly protected against cardiomyocyte necrosis and apoptosis following ischemia-reoxygenation injury. The use of rapamycin and other mTOR inhibitors therefore presents a novel therapeutic strategy to limit myocardial infarction and apoptosis following ischemia/reperfusion injury and attenuate ventricular remodeling, in addition to their well established effect on reduction of post-stent restenosis in humans.

EXAMPLE 1A

1. Introduction

Before this invention, although considerable progress had been made toward identifying cellular triggers and signal transduction mechanisms involved in the process of preconditioning and many pharmacological agents have been shown to

induce a preconditioning like-effect, no agent has yet gained widespread clinical use. Bolli et al., supra.

There is now demonstrated a novel approach of preconditioning the heart by using rapamycin, an increasingly widespread staple of cardiac practice.

Rapamycin (sirolimus) is an antibiotic derived from *Streptomyces hygroscopius*, and for many years has been primarily used as an immunosuppressant in the treatment of organ rejection in transplant recipients (Morris RE. Prevention and treatment of allograft rejection in vivo by rapamycin: molecular and cellular mechanisms of action. *Ann N Y Acad Sci* 1993; 685:68-72.) Recently, rapamycin's anti-growth properties have been utilized for cardiovascular benefit as stents impregnated with rapamycin effectively reduce coronary restonosis (Morice MC, Serruys PW, Sousa JE, Fajadet J, Ban HE, Perin M *et al.* A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. *N Engl J Med* 2002; 346(23):1773-1780.) The proposed mechanism for the anti-proliferative effect of rapamycin is based on its ability to bind to its intracellular receptor, the FK506 binding protein (FKBP12) (Marks AR. Cellular functions of immunophilins. *Physiol Rev* 1996; 76(3):631-649.) The rapamycin/FKBP12 complex is an inhibitor of the mammalian target of rapamycin (mTOR), a 290-kDa Ser/Thr kinase that controls mammalian protein translational processes that are central to cell growth (Schmelzle T, Hall MN. TOR, a central controller of cell growth. *Cell* 2000; 103(2):253-262.) Rapamycin prevents DNA and protein synthesis, in large part, by regulation of p70S6 kinase (p70S6K) phosphatase, leading to arrest of the cell cycle at the G1/S interface (Marx SO, Jayaraman T, Go LO, Marks AR. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. *Circ Res* 1995; 76(3):412-417.) Additionally rapamycin modulation of the mTOR kinase plays key roles in nutrient regulation (Cardenas ME, Cutler NS, Lorenz MC, Di Como CJ, Heitman J. The TOR signaling cascade regulates gene expression in response to nutrients. *Genes Dev* 1999; 13(24):3271-3279), mitochondrial metabolism (Desai BN, Myers BR, Schreiber SL. FKBP12-rapamycin-associated protein associates with mitochondria and senses osmotic stress via mitochondrial dysfunction. *Proc Natl Acad Sci U S A* 2002; 99(7):4319-4324; Edinger AL, Thompson CB. Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol Biol Cell* 2002; 13(7):2276-2288), and growth-factor stimulated proliferation (Marx et al., supra; Mohacsi PJ, Tuller D, Hulliger B, Wijngaard PL. Different inhibitory effects of

immunosuppressive drugs on human and rat aortic smooth muscle and endothelial cell proliferation stimulated by platelet-derived growth factor or endothelial cell growth factor. *J Heart Lung Transplant* 1997; 16(5):484-492.)

In a short time rapamycin-eluting stents have become a vital tool for native coronary artery revascularization and also demonstrate favorable outcomes for treatment of saphenous vein graft lesions (Ge L, Iakovou I, Sangiorgi GM, Chieffo A, Melzi G, Cosgrave J *et al.* Treatment of saphenous vein graft lesions with drug-eluting stents: immediate and midterm outcome. *J Am Coll Cardiol* 2005; 45(7):989-994) and in-stent restonosis (Holmes DR, Jr., Teirstein P, Satler L, Sketch M, O'Malley J, Popma JJ *et al.* Sirolimus-eluting stents vs vascular brachytherapy for in-stent restenosis within bare-metal stents: the SISR randomized trial. *JAMA* 2006; 295(11):1264-1273.) Furthermore, rapamycin taken orally has also shown promise as a potential agent to inhibit restonosis after stenting of de novo coronary lesions (Waksman R, Ajani AE, Pichard AD, Torguson R, Pinnow E, Canos D *et al.* Oral rapamycin to inhibit restenosis after stenting of de novo coronary lesions: the Oral Rapamune to Inhibit Restenosis (ORBIT) study. *J Am Coll Cardiol* 2004; 44(7):1386-1392; Hausleiter J, Kastrati A, Mehilli J, Vogeser M, Zohlnhofer D, Schuhlen H *et al.* Randomized, double-blind, placebo-controlled trial of oral sirolimus for restenosis prevention in patients with in-stent restenosis: the Oral Sirolimus to Inhibit Recurrent In-stent Stenosis (OSIRIS) trial. *Circulation* 2004; 110(7):790-795; Rodriguez AE, Rodriguez AM, Vigo CF, Fernandez PC, Llaurado C, Vetcher D *et al.* Role of oral rapamycin to prevent restenosis in patients with de novo lesions undergoing coronary stenting: results of the Argentina single centre study (ORAR trial). *Heart* 2005; 91(11):1433-1437) as well as slow down the progression of coronary artery disease in heart transplant recipients (Keogh A, Richardson M, Ruygrok P, Spratt P, Galbraith A, O'Driscoll G *et al.* Sirolimus in de novo heart transplant recipients reduces acute rejection and prevents coronary artery disease at 2 years: a randomized clinical trial. *Circulation* 2004; 110(17):2694-2700). Although, the anti-growth effect of rapamycin on vascular tissue has been widely described (Schmelzle, et al., supra), the full spectrum of action of rapamycin, particularly on cardiac tissue, is not known.

Chemical preconditioning with some immunosuppressants has been shown in the heart (Cumming DV, Heads RJ, Coffin RS, Yellon DM, Latchman DS. Pharmacological preconditioning of primary rat cardiac myocytes by FK506. *Basic Res Cardiol* 1996; 91(5):367-373), brain (Sharkey J, Butcher SP. Immunophilins

mediate the neuroprotective effects of FK506 in focal cerebral ischaemia. *Nature* 1994; 371(6495):336-339), and liver (Dhar DK, Nagasue N, Uchida M, Takemoto Y, Yoshimura H, Yamanoi A *et al.* Effective prevention of ischemic injury of the dearterialized canine liver by FK506 pretreatment. *Transplantation* 1993; 56(6):1555-1558), however the cardioprotective effect of rapamycin has been disputed (Kis A, Yellon DM, Baxter GF. Second window of protection following myocardial preconditioning: an essential role for PI3 kinase and p70S6 kinase. *J Mol Cell Cardiol* 2003; 35(9):1063-1071; Jonassen AK, Sack MN, Mjos OD, Yellon DM. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. *Circ Res* 2001; 89(12):1191-1198; Hausenloy DJ, Mocanu MM, Yellon DM. Cross-talk between the survival kinases during early reperfusion: its contribution to ischemic preconditioning. *Cardiovasc Res* 2004; 63(2):305-312; Gross ER, Hsu AK, Gross GJ. Opioid-induced cardioprotection occurs via glycogen synthase kinase beta inhibition during reperfusion in intact rat hearts. *Circ Res* 2004; 94(7):960-966.)

In this example, rapamycin is newly shown to induce a preconditioning-like protective effect in the intact heart and adult cardiomyocyte subjected to ischemia/reperfusion. Furthermore, the results of this example show that rapamycin induced cardioprotection is mediated by opening of the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP} channel).

2. Materials and methods

2.1. Animals

Adult male outbred ICR mice were supplied by Harlan Inc. (Indianapolis, IN). Animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2. Drugs and chemicals

Rapamycin was purchased from Sigma-Aldrich (St. Loius, MO) and was dissolved in DMSO (Sigma-Aldrich) for intraperitoneal injection (final DMSO concentration <1%). Unless specified otherwise, all other chemicals including 5-hydroxydecanoate (5-HD), trypan blue dye and triphenyltetrazolium chloride (TTC) were obtained from Sigma-Aldrich.

2.3. Langendorff isolated perfused heart preparation

The methods for the isolated, perfused mouse heart preparation were previously described in detail (Xi L, Hess ML, Kukreja RC. Ischemic preconditioning

in isolated perfused mouse heart: reduction in infarct size without improvement of post-ischemic ventricular function. *Mol Cell Biochem* 1998; 186(1-2):69-77.) In brief, the mouse was anesthetized with pentobarbital sodium (100 mg/kg) and heparin (33 units IP) and the heart was quickly removed from the thorax and placed in a small dish containing ice-cold perfusate and heparin. The aortic opening was rapidly cannulated and tied on a 20-gauge blunt needle that was connected to a Langendorff perfusion system. After cannulation, the heart was retrogradely perfused at a constant pressure of 55 mmHg with modified Krebs-Henseleit (K-H) solution 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 perfusion solution was continuously gassed with 95% O_2 + 5% CO_2 (pH ~7.4) and warmed by a heating/cooling bath. The heart temperature was continuously monitored and maintained at 37°C throughout the experiment. Ventricular function was measured by a force-displacement transducer (model FT03, Grass) attached to the apex with a no. 5 surgical thread and a rigid metal hook. The resting tension of the isolated heart was adjusted to ~0.30 g. Ventricular developed force was continuously recorded with a PowerLab 8SP computerized data acquisition system connected to the force transducer. Coronary flow rate was calculated by timed collection of the perfusate. The hearts were not paced.

2.4. Experiment protocol for drug pretreatment and cardiac ischemia-reperfusion

As illustrated in Figure 1, mice were injected with either rapamycin (0.25 mg/kg, IP) or volume-matched DMSO (solvent for rapamycin) 30 minutes prior to heart isolation. The hearts were isolated and subjected to 30 min of stabilization on a Langendorff system. During the last 10 minutes of stabilization, the hearts were randomized to either continued K-H buffer perfusion or intracoronary infusion of 100 μM 5-HD (at a previously established dose (Wang L, Cherednichenko G, Hernandez L, Halow J, Camacho SA, Figueredo V *et al.* Preconditioning limits mitochondrial Ca(2+) during ischemia in rat hearts: role of K(ATP) channels. *Am J Physiol Heart Circ Physiol* 2001; 280(5):H2321-H2328) through a side arm of a three-way stopcock connected directly above the aortic cannula with a Harvard microdialysis syringe pump (model 22). The pump speed was set at 0.25 ml/min, which was equivalent to

~15% of the normal coronary flow rate of the heart. This was followed by 20 min of no-flow normothermic global ischemia and finally 30 min of reperfusion.

2.5. Measurement of infarct size

At the end of each experiment, the heart was immediately removed from the Langendorff apparatus, weighed, and frozen at -20°C. The frozen heart was manually cut into seven to eight transverse slices of approximately equal thickness (~0.8 mm) and stained by incubation in 10% TTC for 30 min at room temperature (~22°C). TTC buffer was then replaced with 10% formaldehyde, and the slices were fixed for 4-6 hours before infarct area and risk zone were 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 calculated as a percentage of the risk area.

2.6. Isolation of ventricular myocytes and experimental protocol

The mouse ventricular cardiomyocytes were isolated using an enzymatic technique reported in Das A, Xi L, Kukreja RC. Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis. Essential role of nitric oxide signaling. *J Biol Chem* 2005; 280(13):12944-12955. In brief, the mouse was anesthetized with pentobarbital sodium (100 mg/kg, IP) and heart was quickly removed from the chest. Within 3 min, the aortic opening was cannulated onto a Langendorff perfusion system (Xi et al., supra) and heart was retrogradely perfused (37°C) at a constant pressure of 55 mmHg for ~5 min with a Ca^{2+}-free bicarbonate-based buffer containing (in mM): 120 NaCl, 5.4 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 5.6 glucose, 20 $NaHCO_3$, 10 2,3-butanedione monoxime, and 5 taurine, which was continuously gassed with 95%O_2 + 5%CO_2. The enzymatic digestion was commenced 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 chunks and gently aspirated with a transfer pipette for facilitating the cell dissociation. The cell pellet was resuspended for a 3-step Ca^{2+} restoration procedure (*i.e.* 125, 250, 500 μM Ca^{2+}). The freshly isolated cardiomyocytes were then suspended in minimal essential medium (pH 7.35-7.45) 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 2-chamber slides, which were pre-coated with 20 μg/mL mouse laminin in PBS + 1% penicillin-

streptomycin for 1 hour. The cardiomyocytes were cultured in the presence of 5% CO_2 for 1 hour in a humidified incubator at 37°C, which allowed cardiomyocytes to attach to the slide surface prior to the experimental protocol.

The cultured cardiomyocytes were incubated under 37°C and 5% CO_2, for 1 hour with or without 25, 50 or 100 nM rapamycin. Cardiomyocytes were subjected to simulated ischemia (SI) for 40 minutes by replacing the cell medium with an "ischemia buffer" which contained (in mM): 118 NaCl, 24 $NaHCO_3$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$-$2H_2O$, 1.2 $MgCl_2$, 20 sodium lactate, 16 KCl, 10 2-deoxyglucose (pH adjusted to 6.2) similar to those previously published (Das et al., supra.) In addition, the cells were incubated under hypoxic conditions at 37 °C during the entire SI period by adjusting the tri-gas incubator to 1-2% O_2 and 5% CO_2. Reoxygenation (RO) was accomplished by replacing the ischemic buffer with normal medium under normoxic conditions. Assessment of cell necrosis and apoptosis was performed at 1 hour and 18 hours of RO, respectively.

2.7. Evaluation of cell viability

Cell viability was assessed by trypan blue exclusion assay. After SI and 1 hour of RO, 20 μL of 0.4% trypan blue was added into the culture dish. After ~5 min of equilibration, the cells were counted under microscope.

2.8. TUNEL Staining

Cardiomyocyte apoptosis was analyzed by terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining, using a kit purchased from BD Biosciences, which detects nuclear DNA fragmentation via a fluorescence assay as previously reported (Das et al., supra.) In brief, after SI and 18 hrs of RO, the cells in two chamber slides were fixed by 4% formaldehyde/PBS at 4°C for 25 min and subjected to TUNEL assay according to the manufacturer's protocol.

2.9. Data Analysis and Statistics

The data are presented as means ± SEM. The difference between groups was analyzed by one-way analysis of variance followed by Student-Newman-Keuls post-hoc test. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Baseline cardiovascular function

The adult male ICR mice used in this example weighed an average of 34.7±0.9 g (n=24). There was no significant difference in heart-weight/body-weight

among the groups. Pre-ischemic baseline values of the isolated perfused hearts are summarized in Table 1.

Table 1
Baseline Cardiovascular Function

	Pre-Drug				Post-Drug			
	DMSO	RAPA	DMSO+ 5-HD	RAPA+ 5-HD	DMSO	RAPA	DMSO+ 5-HD	RAPA+ 5-HD
HR (beats/min)	383±29	391±18	368±25	378±33	370±25	408±19	378±24	343±23
DF (g)	0.60±0.07	0.61±0.05	1.02±0.18*	0.53±0.09	0.58±0.06	0.62±0.03	1.08±0.20*	0.35±0.04
RFP (g*beats/min)	226±25	236±20	366±59†	195±34	214±24	251±10†	388±53*	117±14‡
CF (ml/min)	1.62±0.16	1.44±0.11	1.35±0.16	1.87±0.31				

HR, heart rate; DF, developed force; RFP, rate-force product; CF, coronary flow.
*P<0.05 vs. DMSO, RAPA, and RAPA+5-HD (ANOVA).
†P<0.05 vs. RAPA+5-HD (ANOVA).
‡P<0.05 vs. Pre-Drug (paired *t*-test).

Baseline function (developed force and rate-force product) was similar between the RAPA and DMSO pretreated groups. The DMSO pretreated group that received 5-HD infusion during stabilization (DMSO+5-HD) had an elevated developed force prior to 5-HD infusion, compared to the other three groups (P<0.05). However, there was no change in DMSO+5-HD contractility with infusion of 5-HD. In contrast, infusion of 5-HD led to a decrease in rate-force product in the RAPA+5-HD group (P<0.05).

3.2. Rapamycin preconditioning reduced infarct size

Infarct size in the DMSO treated mice was 28.2±1.3% of risk area which was consistent with previously reported results (Wang X, Yin C, Xi L, Kukreja RC. Opening of Ca2+-activated K+ channels triggers early and delayed preconditioning against I/R injury independent of NOS in mice. *Am J Physiol Heart Circ Physiol* 2004; 287(5):H2070-H2077.) Pretreatment with rapamycin reduced infarct size (down to 10.1±2.8%) compared to DMSO controls (a 64% decrease, P<0.001). The degree of infarct size reduction with rapamycin was superior than IPC (Xi et al., supra) and similar to what has been observed with diazoxide (54% decrease), a

selective mitoK_{ATP} channel opener (Ockaili R, Emani VR, Okubo S, Brown M, Krottapalli K, Kukreja RC. Opening of mitochondrial KATP channel induces early and delayed cardioprotective effect: role of nitric oxide. *Am J Physiol* 1999; 277(6 Pt 2):H2425-H2434.) 5-HD infusion during stabilization blocked this protective effect (infarct area 32.2±1.8%, P<0.001 vs. rapamycin), indicating a key role of mitoK_{ATP} channel in rapamycin induced preconditioning.

3.3. Functional Data

Infarct limiting effects of rapamycin was not associated with improved ventricular function (Fig. 3A). Recovery of developed force and rate-force product was similar among all groups. In addition, there was no significant difference in coronary flow from baseline to reperfusion among the groups (Fig. 3B).

3.4. Isolated myocytes data

Investigation was performed of the protective effects of rapamycin on necrosis and apoptosis in adult cardiomyocytes model of simulated ischemia and reoxygenation. Cell preparation was performed and yielded at least 70% of the cardiomyocytes with rod shape morphology, similar to previously reported studies (Das et al., supra.) After 40 min of SI followed by 1 h of RO the number of tryptan blue positive cells increased from 2.9±0.2% in the control group to 33.7±1.2% in the SI-RO group, demonstrating worsening cardiomyocyte necrosis (P<0.001 vs. control). Treatment with rapamycin at each of the doses studied (25, 50, and 100 nM) for 1 hr prior to simulated ischemia decreased the number of tryptan blue positive cells, indicating improved cell viability with rapamycin (P<0.001 vs. SI-RO). For example 33.7±1.2% of total counted cells after SI-RO group were positive for tryptan blue compared to 25.4±0.7% of the cells pretreated with 50 nM rapamycin (P<0.001).

Despite considerable necrosis following 40 min of SI and 1 h of RO, apoptosis was low under these conditions (0.7±0.3% cells with TUNEL-positive nuclei). However, apoptosis was clearly evident after 40 min SI and extended reoxygenation of 18 h (19.6±1.4% cells with TUNEL-positive nuclei, P<0.001 vs. control). Cells treated with rapamycin (25, 50, or 100 nM) 1 h preceding 40 min SI and 18 h RO had markedly decreased number of TUNEL-positive nuclei (10.1±0.7% for 50 nM rapamycin, P<0.001 vs. 19.6±1.4% cells with SI-RO), demonstrating rapamycin protection against apoptotic cell death.

4. Discussion

The use of rapamycin has greatly increased during the past few years with the introduction of rapamycin-eluting coronary stents. While the anti-hypertrophic effects of rapamycin have been well described (Schmelzle, supra), conventionally other properties of this pharmacological agent are poorly understood. The preconditioning-like effect of rapamycin in the mouse heart is a new discovery, disclosure and invention. More specifically, it has now been shown that intraperitoneal administration of rapamycin induces cardioprotection as demonstrated by significant reduction in infarct size. Furthermore, these finding have been replicated at the cellular level by showing that rapamycin directly protected against cardiomyocyte necrosis and apoptosis following ischemia-reoxygenation injury. Although rapamycin has a complex mechanism of action, the results herein further showed that the protective effect of this agent was abolished by 5-HD thereby suggesting the essential role of mitoK_{ATP} channel in protection. To our knowledge, this is the first investigation that demonstrates the preconditioning-like effect of rapamycin against infarction in the intact heart as well against necrosis and apoptosis in isolated adult cardiomyocytes.

4.1 Potential mechanisms

The experimental findings of this example challenge the precept previously set forth by others that rapamycin can only inhibit cardioprotection and in itself has no protective effects. Indeed, several studies have established that IPC (Kis et al., supra; Hausenloy et lal, supra), insulin (Janassen et al., supra), and opioids (Gross et al,. supra) initiate the PI3K-Akt signaling cascade and confer cardioprotection through recruitment of mTOR-p70S6K. Rapamycin in these studies blocked early preconditioning (Gross et al., supra), delayed preconditioning (Kis et al., supra), as well as, protection at reperfusion (Jonassen et al., supra; Hausenloy et al., supra). Thus others' findings at first seem divergent from the inventor's observation of a potent preconditioning like-effect of rapamycin. However there are some clear explanations that reconcile these seemingly conflicting differences.

First of all, rapamycin mediated cardioprotection is easily explained when viewing the survival kinase cascade as a complex process with "cross-talk" between the kinases – as inhibition of one pathway upregulates upstream signaling elsewhere. In support of this concept, Shi et al. observed in multiple myeloma cells that mTOR inhibition with rapamycin upregulated PI3K activity as well as Akt activity and

phosphorylation (Shi Y, Yan H, Frost P, Gera J, Lichtenstein A. Mammalian target of rapamycin inhibitors activate the AKT kinase in multiple myeloma cells by up-regulating the insulin-like growth factor receptor/insulin receptor substrate-1/phosphatidylinositol 3-kinase cascade. *Mol Cancer Ther* 2005; 4(10):1533-1540.) O'Reilly and colleagues replicated these findings in a variety of tumor cell lines (Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D *et al.* mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 2006; 66(3):1500-1508.) Interestingly, findings from both groups demonstrated that upregulation of PI3K-Akt was due to a suppression of the feedback inhibition on IGF-1. mTOR-Akt "cross-talk" has also been confirmed in cardiomyocytes as Li et al. reported that rapamycin significantly enhanced Akt phosphorylation (Li SY, Fang CX, Aberle NS, Ren BH, Ceylan-Isik AF, Ren J. Inhibition of PI-3 kinase/Akt/mTOR, but not calcineurin signaling, reverses insulin-like growth factor I-induced protection against glucose toxicity in cardiomyocyte contractile function. *J Endocrinol* 2005; 186(3):491-503.) Yellon and colleagues also demonstrated "cross-talk" between the survival kinases in rat hearts as PI3K-Akt inhibition at reperfusion induced the phosphorylation of Erk1/2-p70S6K, and conversely, that MEK1/2-Erk1/2 inhibition induced the phosphorylation of Akt (Hausenloy et al., supra). Although the study by Yellon and colleagues (Hausenloy et al., supra) did not comment on the effect of mTOR inhibition on other kinases, the group in an earlier report observed no change in Akt phosphorylation with rapamycin (Kis et al., supra.) Although speculative, it is possible that the reduction in infarct size observed in our study is due to mTOR upregulation of other survival kinases, including Akt. In addition, p70S6K has two different isoforms, α and β, with different response to rapamycin (Minami T, Hara K, Oshiro N, Ueoku S, Yoshino K, Tokunaga C *et al.* Distinct regulatory mechanism for p70 S6 kinase beta from that for p70 S6 kinase alpha. *Genes Cells* 2001; 6(11):1003-1015) and it is easy to conceive of some p70S6K activation can occur through rapamycin-insensitive forms.

Another compelling explanation for the observed cardioprotection in our study may well be due to the inhibition of protein synthesis through mTOR-dependent signaling. A previous study has shown that inhibition of mitochondrial protein synthesis by chloramphenicol significantly reduced infarct size during ischemia-reperfusion injury (He H, Chen M, Scheffler NK, Gibson BW, Spremulli LL, Gottlieb RA. Phosphorylation of mitochondrial elongation factor Tu in ischemic myocardium:

basis for chloramphenicol-mediated cardioprotection. *Circ Res* 2001; 89(5):461-467.) Specifically, He et al. found that IPC modulated the phosphorylation of mitochondrial translational elongation factor, which is known to inhibit protein synthesis. Similarly, another protein synthesis inhibitor, anisomycin has also been shown to induce acute and delayed preconditioning effect in the heart, mediated by opening of mitoK_{ATP} (Baines CP, Liu GS, Birincioglu M, Critz SD, Cohen MV, Downey JM. Ischemic preconditioning depends on interaction between mitochondrial KATP channels and actin cytoskeleton. *Am J Physiol* 1999; 276(4 Pt 2):H1361-H1368; Zhao TC, Taher MM, Valerie KC, Kukreja RC. p38 Triggers late preconditioning elicited by anisomycin in heart: involvement of NF-kappaB and iNOS. *Circ Res* 2001; 89(10):915-922.)

Although the role of endogenous nitric oxide (NO) during acute phase of IPC is not resolved, there is gathering evidence that exogenous NO is cardioprotective (Nakano A, Liu GS, Heusch G, Downey JM, Cohen MV. Exogenous nitric oxide can trigger a preconditioned state through a free radical mechanism, but endogenous nitric oxide is not a trigger of classical ischemic preconditioning. *J Mol Cell Cardiol* 2000; 32(7):1159-1167.) It has been recently demonstrated that NO generation from endothelial and inducible NO synthase (eNOS and iNOS) stimulates guanylyl cyclase to produce cGMP, which in turn activates protein kinase G and finally leads to opening of mitoK_{ATP} (Dang VC, Kim N, Youm JB, Joo H, Warda M, Lee JH *et al.* Nitric oxide-cGMP-protein kinase G signaling pathway induces anoxic preconditioning through activation of ATP-sensitive K+ channels in rat hearts. *Am J Physiol Heart Circ Physiol* 2005.) The opening of mitoK_{ATP} channel further activates downstream kinases to promote cell survival signaling (Yellon et al., supra.) In this respect, the potential role of NO in rapamycin-induced protection may be worthy of consideration. Recent studies have shown that rapamycin treatment over 10 weeks significantly increased eNOS expression in $ApoE^{-/-}$ mice (Naoum JJ, Zhang S, Woodside KJ, Song W, Guo Q, Belalcazar LM *et al.* Aortic eNOS expression and phosphorylation in Apo-E knockout mice: differing effects of rapamycin and simvastatin. *Surgery* 2004; 136(2):323-328.) Additionally, rapamycin, but not cyclosporine, treatment inhibited the development of intimal hyperplasia and increased the expression of iNOS in a rat aortic allograft model (Pham SM, Shears LL, Kawaharada N, Li S, Venkataramanan R, Sehgal S. High local production of nitric oxide as a possible mechanism by which rapamycin prevents transplant

arteriosclerosis. *Transplant Proc* 1998; 30(4):953-954.)

4.2 Role of Mitochondrial K_{ATP} Channels

The results of this example demonstrate that rapamycin induced cardioprotection is mediated by opening of mitoK_{ATP} channel as inhibition of the channel by 5-HD abrogated the infarct sparing effect of rapamycin. Several previous studies have shown that opening the mitoK_{ATP} channel is one of the common mediators of acute and delayed preconditioning, induced by both pathophysiological stressors (Schultz JE, Qian YZ, Gross GJ, Kukreja RC. The ischemia-selective KATP channel antagonist, 5-hydroxydecanoate, blocks ischemic preconditioning in the rat heart. *J Mol Cell Cardiol* 1997; 29(3):1055-1060; Liu Y, Sato T, Seharaseyon J, Szewczyk A, O'Rourke B, Marban E. Mitochondrial ATP-dependent potassium channels. Viable candidate effectors of ischemic preconditioning. *Ann N Y Acad Sci* 1999; 874:27-37; Gross GJ, Peart JN. KATP channels and myocardial preconditioning: an update. *Am J Physiol Heart Circ Physiol* 2003; 285(3):H921-H930; O'Rourke B. Evidence for mitochondrial K+ channels and their role in cardioprotection. *Circ Res* 2004; 94(4):420-432) as well as pharmacological agents (Fryer RM, Hsu AK, Eells JT, Nagase H, Gross GJ. Opioid-induced second window of cardioprotection: potential role of mitochondrial KATP channels. *Circ Res* 1999; 84(7):846-851; Ockaili RA, Bhargava P, Kukreja RC. Chemical preconditioning with 3-nitropropionic acid in hearts: role of mitochondrial K(ATP) channel. *Am J Physiol Heart Circ Physiol* 2001; 280(5):H2406-H2411; Wang Y, Kudo M, Xu M, Ayub A, Ashraf M. Mitochondrial K(ATP) channel as an end effector of cardioprotection during late preconditioning: triggering role of nitric oxide. *J Mol Cell Cardiol* 2001; 33(11):2037-2046; Ockaili R, Salloum F, Hawkins J, Kukreja RC. Sildenafil (Viagra) induces powerful cardioprotective effect via opening of mitochondrial K(ATP) channels in rabbits. *Am J Physiol Heart Circ Physiol* 2002; 283(3):H1263-H1269.) Opening of mitoK_{ATP} channel during the IPC phase leads to the generation of reactive oxygen species (ROS) (Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS *et al.* Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ Res* 2000; 87(6):460-466; Forbes RA, Steenbergen C, Murphy E. Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. *Circ Res* 2001; 88(8):802-809; Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* 1998;

273(29):18092-18098.) ROS then act as a second messenger to activate the downstream pathway of protective kinases, including protein kinase C and others (Oldenburg O, Cohen MV, Downey JM. Mitochondrial K(ATP) channels in preconditioning. *J Mol Cell Cardiol* 2003; 35(6):569-575). This small burst of ROS generated by mitoK_{ATP} channel prior to ischemia acts to prevent the larger, damaging burst during reperfusion (Liu et al., supra). There are some explanations of how rapamycin may open mitoK_{ATP} channel. First of all, several studies have demonstrated that rapamycin induced mTOR inhibition enhances compensatory upregulation of upstream survival kinases, such as PI3K and Akt (Gursoy et al., supra; Oldenburg et al., supra; Shi et al., supra.) These kinases in turn are key mediators in the activation of mitoK_{ATP} channel (Forbes et al., supra.) In addition, it is certainly possible that spatial co-localization of mTOR with the mitochondria allows for physiological regulation of mitochondrial membrane channel activity (Desai et al., supra.) Furthermore, rapamycin may upregulate NO and several studies have found that NO consequently plays an important role in the opening of mitoK_{ATP} channel in cardiomyocytes (Ockaili et al., supra; Sasaki N, Sato T, Ohler A, O'Rourke B, Marban E. Activation of mitochondrial ATP-dependent potassium channels by nitric oxide. *Circulation* 2000; 101(4):439-445.)

4.3 Apoptosis

The observation in this example that rapamycin inhibits cardiomyocyte necrosis and apoptosis in myocytes further supports the infarct limiting effects of this drug in the intact heart. The precise influence of rapamycin on apoptosis continues to be vigorously debated with seemingly divergent results observed in multiple cell lines. Rapamycin blocked Akt-p70S6K mediated survival during stress-induced apoptosis in HUVEC (Joshi MB, Philippova M, Ivanov D, Allenspach R, Erne P, Resink TJ. T-cadherin protects endothelial cells from oxidative stress-induced apoptosis. *FASEB J* 2005; 19(12):1737-1739) and B-lymphocytic cells (Edinger et al., supra). Hypoxia-inducible factor-lα (HIF-1α) (Majumder PK, Febbo PG, Bikoff R, Berger R, Xue Q, McMahon LM *et al.* mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. *Nat Med* 2004; 10(6):594-601; Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol* 2001; 21(12):3995-4004), elF4E

(Wendel HG, De Stanchina E, Fridman JS, Malina A, Ray S, Kogan S *et al.* Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature* 2004; 428(6980):332-337) and c-JUN (Huang S, Shu L, Dilling MB, Easton J, Harwood FC, Ichijo H *et al.* Sustained activation of the JNK cascade and rapamycin-induced apoptosis are suppressed by p53/p21(Cip1). *Mol Cell* 2003; 11(6):1491-1501) have been reported as the downstream effectors for the apoptotic effect of mTOR. In contrast, rapamycin has been shown to exert anti-apoptotic effects in B-cell lymphoma (Calastretti A, Rancati F, Ceriani MC, Asnaghi L, Canti G, Nicolin A. Rapamycin increases the cellular concentration of the BCL-2 protein and exerts an anti-apoptotic effect. *Eur J Cancer* 2001; 37(16):2121-2128), HEK (Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 2003; 115(5):577-590), multiple myeloma (Thyrell L, Hjortsberg L, Arulampalam V, Panaretakis T, Uhles S, Dagnell M *et al.* Interferon alpha-induced apoptosis in tumor cells is mediated through the phosphoinositide 3-kinase/mammalian target of rapamycin signaling pathway. *J Biol Chem* 2004; 279(23):24152-24162), and Jurkat cells (Fumarola C, La Monica S, Alfieri RR, Borra E, Guidotti GG. Cell size reduction induced by inhibition of the mTOR/S6K-signaling pathway protects Jurkat cells from apoptosis. *Cell Death Differ* 2005; 12(10):1344-1357). The current results are in agreement with anti-apoptotic effect of rapamycin in the adult cardiomycytes, although the exact mechanism needs to be further investigated. Certainly rapamycin's interaction with the mitoK_{ATP} channel may contribute in the inhibition of apoptosis. A previous report by Akao et al. has shown that openers of the mitoK_{ATP} channel were able to reduce apoptosis induced by oxidative stress in the neonatal rat cardiomyocyte (Akao M, Ohler A, O'Rourke B, Marban E. Mitochondrial ATP-sensitive potassium channels inhibit apoptosis induced by oxidative stress in cardiac cells. *Circ Res* 2001; 88(12):1267-1275.)

In addition to its anti-apoptotic activity, rapamycin may have potential effects on myocyte proliferation. Several labs have documented that the heart has an endogenous reserve of progenitor cells that have the ability to proliferate and potentially reconstitute infracted myocardium (Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S *et al.* Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 2003; 114(6):763-776; Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y *et al.* Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl*

Acad Sci U S A 2003; 100(21):12313-12318; Pfister O, Mouquet F, Jain M, Summer R, Helmes M, Fine A *et al.* CD31- but not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. *Circ Res* 2005; 97(1):52-61; Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* 2004; 95(9):911-921; Martin CM, Meeson AP, Robertson SM, Hawke TJ, Richardson JA, Bates S *et al.* Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. *Dev Biol* 2004; 265(1):262-275; Linke A, Muller P, Nurzynska D, Casarsa C, Torella D, Nascimbene A *et al.* Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. *Proc Natl Acad Sci U S A* 2005; 102(25):8966-8971). Recently Urbanek et al. have documented the activation, proliferation, and migration of these cells by HGF and IGF-1 (Urbanek K, Rota M, Cascapera S, Bearzi C, Nascimbene A, De Angelis A *et al.* Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. *Circ Res* 2005; 97(7):663-673). This is of particular interest because rapamycin has been shown to upregulate PI3K (a critical effector in HGF mediated migration) and also likely enhance IGF-1 activity (Gursoy et al., supra; Oldenburg et al., supra; Shi et al., supra).

4.4 Dosage/concentration

It is noted that Gross et al. showed that a smaller dose of rapamycin (3 μg/kg IV) did not confer cardioprotection in their intact rat model of ischemia (Gross et al., supra). The concentration used in the isolated myocyte experiments of this example was chosen because rapamycin at 25-100 nM has previously been shown to selectively inhibit mTOR activity without affecting other protein kinases (Chung J, Kuo CJ, Crabtree GR, Blenis J. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* 1992; 69(7):1227-1236; Price DJ, Grove JR, Calvo V, Avruch J, Bierer BE. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. *Science* 1992; 257(5072):973-977; Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 2000; 351(Pt 1):95-105). Specifically, Chung et al., studying a variety of cell lines,

demonstrated that rapamycin maximally inhibited activation of p70S6K between 4 to 20 nM, without affecting serum stimulated activation of MAPK or a distinct family of S6 kinases. In intact mice the dosages commonly used in the literature range from as low as 3μg/kg IV (Gross et al., supra) to 2-3 mg/kg IP (Shioi T, McMullen JR, Tarnavski O, Converso K, Sherwood MC, Manning WJ *et al.* Rapamycin attenuates load-induced cardiac hypertrophy in mice. *Circulation* 2003; 107(12):1664-1670; Poston RS, Billingham M, Hoyt EG, Pollard J, Shorthouse R, Morris RE *et al.* Rapamycin reverses chronic graft vascular disease in a novel cardiac allograft model. *Circulation* 1999; 100(1):67-74.) In this experiment, 0.25 mg/kg was used in the intact mice because Kis et al. recently confirmed that rapamycin inhibits p70S6K activity at this dose.

4.5 Conclusion

In summary, it has been demonstrated, for the first time, that rapamycin induces preconditioning-like protective effects against myocardial infarction following ischemia-reperfusion injury through opening of mitoK_{ATP} channels. In addition, this drug reduced necrosis as well as apoptosis following simulated ischemia-reoxygenation injury in adult cardiomyocytes. Rapamycin therefore may be used as a novel therapeutic strategy to limit myocardial infarction and apoptosis following ischemia/reperfusion injury and attenuate ventricular remodeling, in addition to its well-established effect on reduction of post-stent restenosis in humans.

EXAMPLE 2

(Rapamycin (Sirolimus) Administration *in vivo* Limits Post-Ischemic Myocardial Injury by MAPK Signaling in the Mouse Heart)

Background: The therapeutic application of rapamycin, a potent inhibitor of downstream signaling from the mammalian target of rapamycin (mTOR) proteins, to drug eluting stents significantly reduced the degree of arterial restenosis. However, before this invention, the effect of this antibiotic on cardiac tissue has not been fully examined. This example studies whether acute inhibition of mTOR with rapamycin prior to ischemia/reperfusion (I/R) would attenuate myocardial infarction. This example also studied whether, similar to ischemic preconditioning, mitogen activated protein kinases (MAPK) are involved in this protection.

Background and Methodology

Introduction: In another example, it was shown that pretreatment with

rapamycin reduced infarct size (10.1±2.8%) as compared to DMSO controls (64% decrease, P<0.001) in a Langenforff isolated buffer perfused mouse heart. In this example, it was also demonstrated that after 40 min of simulated ischemia (SI) followed by 1 h of reoxygenation (RO), the number of tryptan blue positive cells increased from 2.9±0.2% in the control group to 33.7±1.2% in the SI-RO group, demonstrating worsening cardiomyocyte necrosis (P<0.001 vs. control). Treatment with rapamycin at each of the doses studied (25, 50, and 100 nM) for 1 hr prior to simulated ischemia decreased the number of tryptan blue positive cells, indicating improved cell viability with rapamycin (P<0.001 vs. SI-RO). For example 33.7±1.2% of total counted cells after SI-RO group were positive for trypan blue compared to 25.4±0.7% of the cells pretreated with 50 nM rapamycin (P<0.001). The purpose of this study was 1- to show if systemic administration of rapamycin reduces infarct size in an in vivo mouse model of regional ischemia/reperfusion injury and 2- to examine the role of MAPK activation in this protection.

Experimental Groups:

The following three groups were used. 1- Controls (mice injected *ip* with normal saline); 2- Rapamycin (0.25 mg/kg; *ip*) administered 30 min. prior to I/R; 3- DMSO (mice injected *ip* with DMSO, the solvent for rapamycin). Six mice in each group were used for infarct size assessment. Three mice per group from groups 2 and 3 were used for western blotting.

Myocardial Infarction Protocol:

A total of 24 male ICR mice (Body weight: 27-33g) were used. The animals were anesthetized with an intraperitoneal injection of pentobarbital (70 mg/kg). The animals were 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. The surgery was carried out under sterile conditions. A left thoracotomy was performed at the fourth intercostal space and the heart was exposed by stripping the pericardium to identify the left coronary artery branch. A ligature was then placed around the left coronary artery, and the artery was occluded by snaring with a small tube through which the ligature had been passed. After 30 minutes of ischemia, the ligature was released and the air was expelled from the chest and the surgical wounds were sutured closed. The animals were observed during recovery until fully conscious and then extubated. The animals received intramuscular doses of analgesia

(buprenorphine 0.02 mg/kg) and antibiotic (Gentamicin 0.7 mg/kg). The hearts we allowed to reperfuse 24 hours before explantation.

Infarct Size:

Myocardial ischemic damage was measured using infarct size which was determined by computer morphometry of 10% triphenyl tetrazolium chloride (TTC) stained heart sections.

Evaluation of MAPK Phosphorylation:

Activation of ERK and JNK MAPK after rapamycin administration was evaluated by Western Blot.

Methods & Results: Adult ICR mice (28-33g) were injected *i.p.* with rapamycin (0.25 mg/kg) or vehicle 30 min prior to regional ischemia by left coronary artery ligation for 30 min and reperfusion for 24 hr. At the end of reperfusion, hearts were collected for infarct size (IS) measurement using computer morphometry of tetrazolium chloride stained sections. Myocardial IS (mean ± SE) was significantly reduced in mice pretreated with rapamycin (65% decline). Risk area was not different between groups. Moreover, WB analysis showed that rapamycin treatment caused an increase in pERK and pJNK 45 min later without affecting total ERK or JNK proteins.

Data: The data obtained provide evidence that acute systemic administration of rapamycin is effective against I/R injury through MAPK signaling. These data suggest that rapamycin may be a useful therapeutic tool to suppress I/R injury in patients with cardiovascular disease.

It was observed that post-ischemic myocardial infarct size was reduced in mice treated with Rapamycin (14.9 ± 2.0%) versus saline control group (40.8 ± 1.5%) and DMSO (43.0 ± 2.5%). There was no significant difference in the risk areas between the groups.

Sections of TTC stained hearts were considered. It was found that rapamycin reduces infarct size following I/R as compared with Saline control. This protection was completely abolished in DMSO treated animals.

Changes in pERK and pJNK Protein 30 min after treatment were evaluated. Western Blot and densitometric analyses showed significant increase in pERK and pJNK protein levels 30 min post *ip* injection with rapamycin or DMSO in mice. Total ERK and total JNK protein levels were not changed.

Normal isolated cardiac myocytes were compared to myocytes subjected to 40 min of simulated ischemia (SI) and 1 h of reoxygenation (RO). Cell necrosis was evident by the increased number of tryptan-positive blue myocytes. Pretreatment with 100 nM rapamycin was found to reduce the number of tryptan-blue positive myocytes demonstrating improved cell viability.

Pretreatment with rapamycin (25, 50, or 100 nM) for 1 h prior to simulated ischemia was found to have decreased the number of trypan blue-positive cells, indicating improved cell viability with rapamycin.

The experimental results of this example demonstrated that rapamycin induces preconditioning-like protective effects against myocardial infarction following ischemia-reperfusion injury through MAPK signaling. This novel cardioprotective effect against ischemia/reperfusion injury is of great clinical interest because this compound is already being used in the clinical arena in transplant medicine and as a coating for drug-eluting stents. This example supports the use of rapamycin as a therapeutic strategy to limit myocardial infarction and apoptosis following ischemia/reperfusion injury and attenuate ventricular remodeling.

EXAMPLE 3 (cardio-protection)

For a person whose heart is at risk (such as, e.g., a person who suffered a heart attack, a person at high risk of cardiovascular disease, etc.) cardioprotection may be undertaken by administration of rapamycin or a rapamycin analogue, such as administration via intraperitoneal route.

EXAMPLE 3A (protection of brain tissue)

For a person whose brain tissue is at risk (such as, e.g., a person who has suffered head trauma, a person at high risk of cardiovascular disease, a person who suffered stroke, etc.) protection of brain tissue may be undertaken by administration of rapamycin or a rapamycin analogue, such as administration via intraperitoneal route.

EXAMPLE 3B (protection of liver tissue)

For a person whose liver tissue is at risk for ischemia/reperfusion injury during hepatic surgical intervention, protection of liver tissue may be undertaken by administration of rapamycin or a rapamycin analogue, such as administration via intraperitoneal route.

EXAMPLE 3C (protection of kidney tissue)

For a person whose kidney tissue is at risk (such as e.g., during ischemic acute

renal failure), protection of kidneytissue may be undertaken by administration of rapamycin or a rapamycin analogue, such as administration via intraperitoneal route.

EXAMPLE 3D (protection of lung tissue)

For a person whose lung tissue is at risk of ischemia/reperfusion injury (such as e.g., during lung transplantation), protection of lung tissue may be undertaken by administration of rapamycin or a rapamycin analogue, such as administration via intraperitoneal route.

EXAMPLE 3E (protection of intestingal tissue)

For a person whose gut tissue is at risk (such as e.g., systemic inflammatory response syndrome), protection of gut tissue may be undertaken by administration of rapamycin or a rapamycin analogue, such as administration via intraperitoneal route.

EXAMPLE 3F (protection of skeletal muscle)

For a person whose skeletal muscle is at risk (such as e.g., peripheral vascular disease), protection of skeletal muscle may be undertaken by administration of rapamycin or a rapamycin analogue, such as administration via intraperitoneal route.

EXAMPLE 3G (protection of spleen tissue)

For a person whose spleen tissue is at risk (such as e.g., acute splenic infarction), protection of spleen tissue may be undertaken by administration of rapamycin or a rapamycin analogue, such as administration via intraperitoneal route.

EXAMPLE 3H (protection of pancreatic tissue)

For a person whose pancreatic tissue is at risk (such as e.g., clinical pancreas transplantation), protection of pancreatic tissue may be undertaken by administration of rapamycin or a rapamycin analogue, such as administration via intraperitoneal route.

EXAMPLE 3I (protection of retinal tissue)

For a person whose retinal tissue is at risk (such as e.g., macular edema, capillary nonperfusion, retinal neovascularization, vitreous hemorrhage, and tractional retinal detachments that often result in loss of vision), protection of retinal tissue may be undertaken by administration of rapamycin or a rapamycin analogue, such as administration via intraperitoneal route.

While the invention has been described in terms of its preferred embodiments, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the appended claims.

CLAIMS

I claim:

1. A method of protecting against necrosis and/or apoptosis in cardiomyocytes, comprising:

administering to a patient having a population of cardiomyocytes an effective dose of a protective substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor.

2. The method of claim 1, wherein the administering step is via an intraperitoneal administration route.

3. A method of preconditioning a patient against myocardial infarction, comprising:

opening mitochondrial KATP channels in the patient by administering to the patient an effective amount of a substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor.

4. The method of claim 3, wherein the administering step is via an intraperitoneal administration route.

5. The method of claim 3, wherein the substance is administered to the patient following ischemia/reperfusion injury in the heart.

6. The method of claim 5, wherein the ischemia/reperfusion injury is an ischemia/reperfusion injury resulting from coronary bypass surgery and/or angioplasty in the heart.

7. A cardio-protective method in a post-heart attack patient, comprising:

after the patient has experienced a heart attack of a heart muscle of the patient, administering to the patient an effective amount of a cardio-protective

substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor.

8. The method of claim 7, wherein the administering step is via an intraperitoneal administration route.

9. The method of claim 8, wherein the amount of the cardio-protective substance administered is an amount sufficient to protect against ischemia/reperfusion injury.

10. The method of claim 9, wherein the ischemia/reperfusion injury results from coronary bypass surgery and/or angioplasty in the heart.

11. The method of claim 7, wherein, after the administering step, the heart muscle undergoes ischemia and/or reperfusion having a less-damaging effect to the heart muscle than if the cardio-protective substance had not been administered.

12. The method of claim 7, comprising a step of limiting infarct effect on the heart muscle, provided that the infarct limiting step comprises a step other than improving ventricular function.

13. A protective method in an at-risk patient, comprising:

administering to the patient an effective amount of a protective substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor, and thereby

protecting a tissue selected from the group consisting of: heart, brain, liver, kidney, lung, gut, skeletal muscle, pancreas, retina and intestinal tissue wherein the protecting comprises reducing an effect to the tissue from ischemia/reperfusion injury.

14. The protective method of claim 13, wherein the at-risk patient is selected from the group consisting of a post-heart attack patient; a patient who has suffered head

trauma; a patient at risk of cardiovascular disease and a patient who has suffered stroke.

15. A protective method in a post-heart attack patient, comprising:

administering to a patient who has experienced a heart attack an amount of a substance selected from the group consisting of: rapamycin; everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor, wherein the amount is an amount effective to prevent a process of heart remodeling.

16. A method of screening whether to administer to a patient a substance selected from the group consisting of rapamycin, everolimus; a rapamycin analogue; and a mammalian target of rapamycin (mTOR) inhibitor, comprising:

determining whether the patient has a factor selecting from the group consisting of having had a heart attack, having suffered head trauma, being at risk of cardiovascular disease, having suffered stroke, having heart tissue at risk of ischemic/reperfusion injury, having brain tissue at risk of ischemia/reperfusion injury, having liver tissue at risk of ischemic/reperfusion injury, having kidney tissue at risk of ischemic/reperfusion injury, having lung tissue at risk of ischemic/reperfusion injury, having gut tissue at risk of ischemic/reperfusion injury, having skeletal muscle at risk of ischemic/reperfusion injury, having spleen tissue at risk of ischemic/reperfusion injury, having pancreatic tissue at risk of ischemic/reperfusion injury, having retinal tissue at risk of ischemic/reperfusion injury and havingintestinal tissue at risk of ischemic/reperfusion injury.

17. The screening method of claim 16, including, if the patient is determined to have at least one such factor, administering the substance to the patient following the determination that the patient has had at least one such factor.

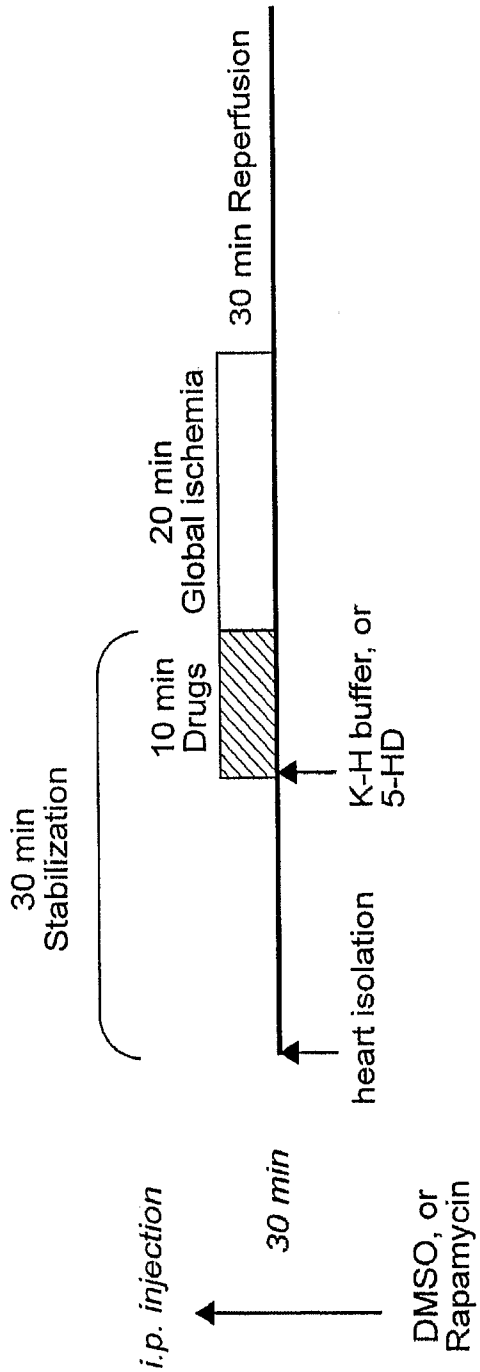
30 min Stabilization
10 min Drugs
20 min Global ischemia
30 min Reperfusion
K-H buffer, or 5-HD
heart isolation
30 min
i.p. injection
DMSO, or Rapamycin

Figure 1

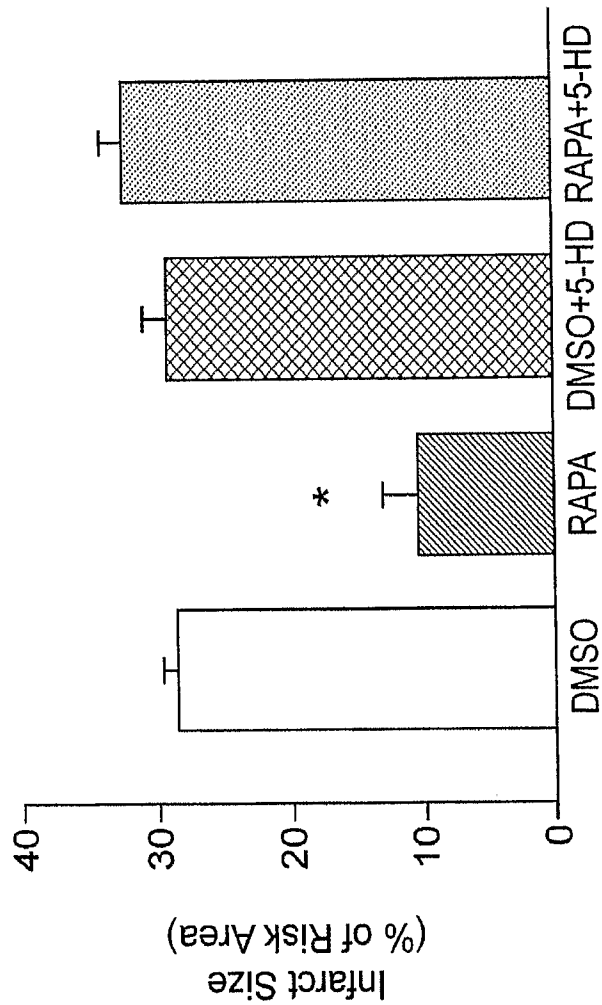
Infarct Size
(% of Risk Area)
40
30
20
10
0
*
DMSO
RAPA
DMSO+5-HD
RAPA+5-HD

Figure 2

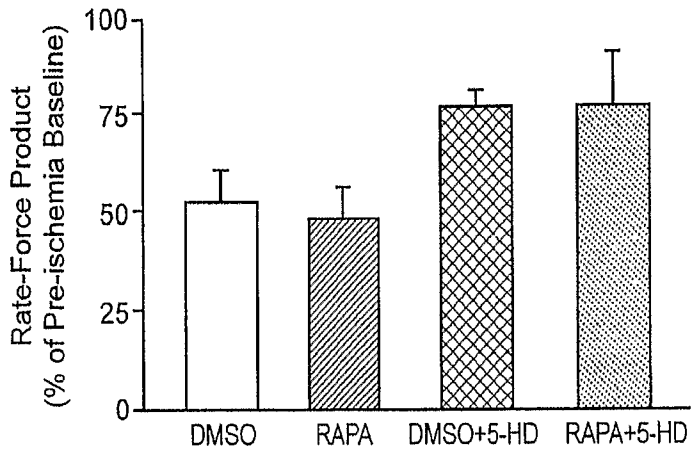

Figure 3A

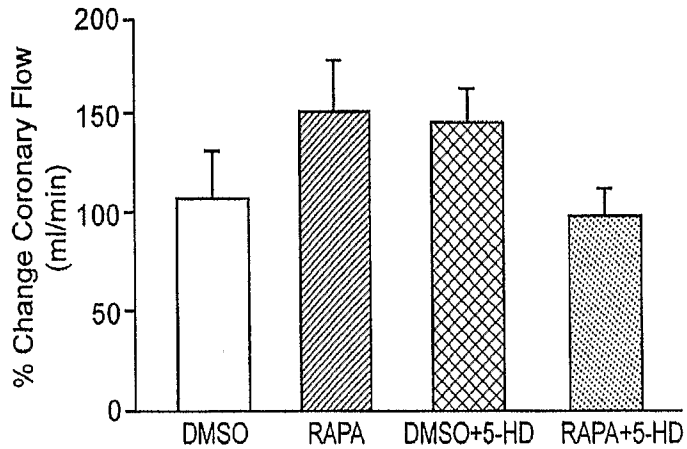

Figure 3B

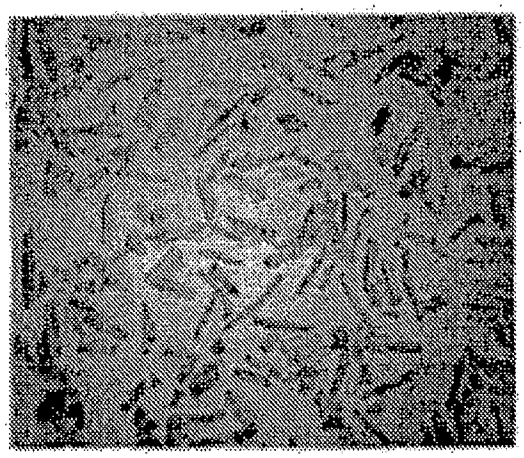

Figure 4A

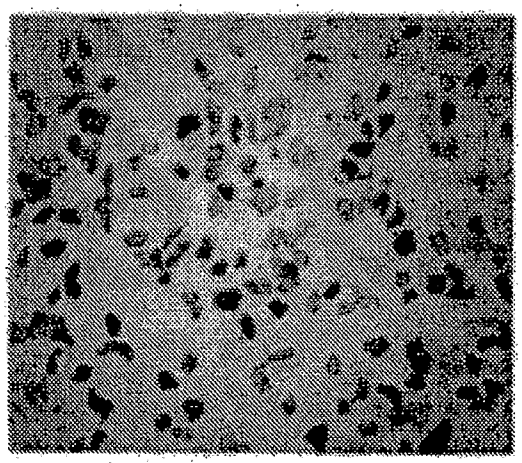

Figure 4B

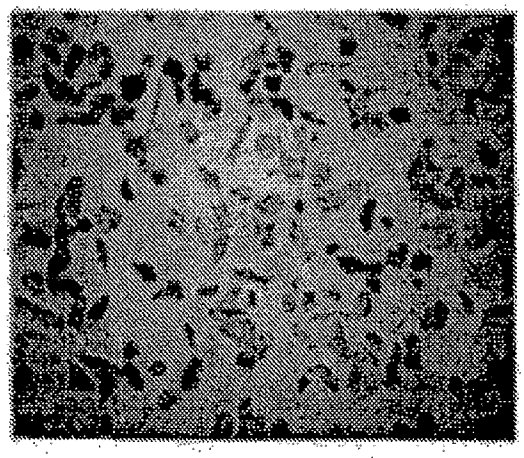

Figure 4C

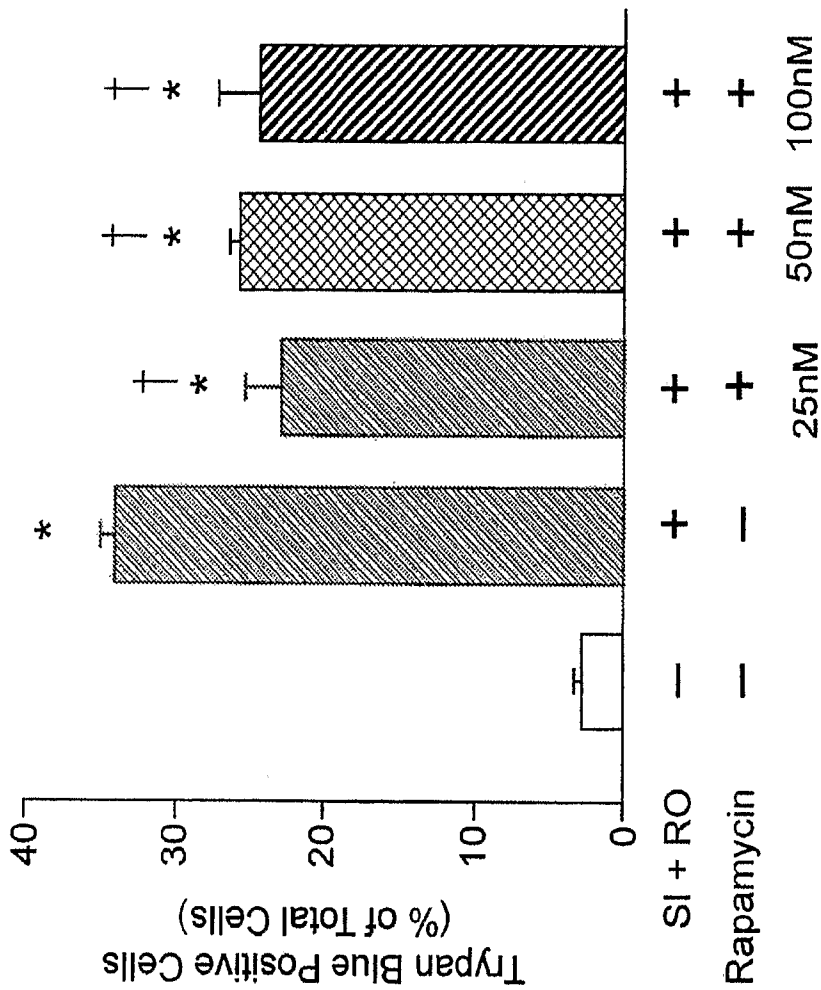
Trypan Blue Positive Cells
(% of Total Cells)
0
10
20
30
40
SI + RO
Rapamycin
25nM
50nM
100nM
*

Figure 5

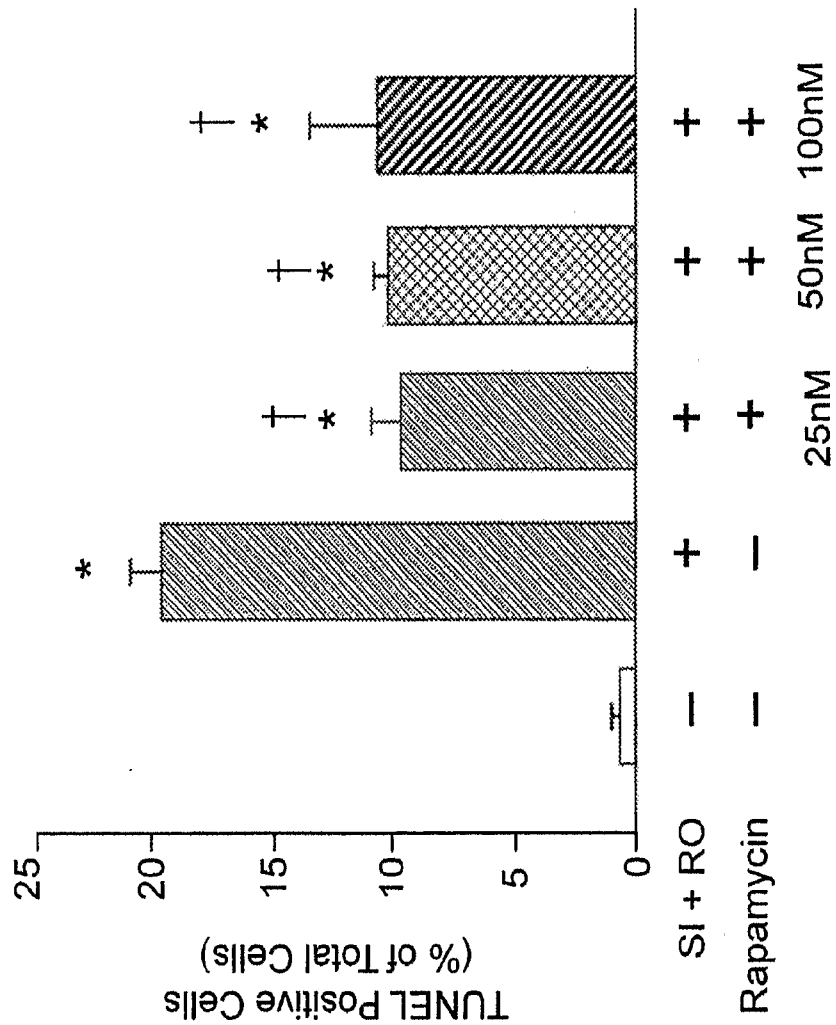
TUNEL Positive Cells
(% of Total Cells)
25
20
15
10
5
0
SI + RO
Rapamycin
25nM
50nM
100nM
*

Figure 6