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PATHOGENIC ROLE OF PHOSPHODIESTERASE TYPE 5 UPREGULATION IN CARDIAC ISCHEMIA/REPERFUSION INJURY

Daniel Hobbs
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PATHOGENIC ROLE OF PHOSPHODIESTERASE TYPE 5 UPREGULATION IN CARDIAC ISCHEMIA/REPERFUSION INJURY

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

by

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List of Abbreviations

Akt	protein kinase B
AMI	acute myocardial infarction
AngII	angiotensin II
ANP	atrial natriuretic peptide
AP-1	activator protein-1
bax	Bcl-2–associated X protein
Bcl2	B-Cell Lymphoma-2
BNP	B-type natriuretic peptide
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
CHD	coronary heart disease
CHF	congestive heart failure
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FPASMC	fetal pulmonary artery smooth muscle cells
H_2O_2	hydrogen peroxide
iNOS	inducible nitric oxide synthase
IP	ischemic preconditioning
I/R	ischemia/reperfusion
K-H	Krebs-Henseleit
LAD	left anterior descending coronary artery
LDH	lactate dehydrogenase
MPG	N-2-mercaptopropionyl glycine
MPTP	mitochondrial permeability transition pore
NO	nitric oxide

NP	natriuretic peptide
p70S6K	protein 70S6 kinase
PDE	3',5'-cyclic nucleotide phosphodiesterase
PDE2	phosphodiesterase type 2
PDE3	phosphodiesterase type 3
PDE5	phosphodiesterase type 5
PDE6	phosphodiesterase type 6
pGC	particulate guanylate cyclase
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
pPDE5	phosphorylated phosphodiesterase type 5
pVASP	phosphorylated-vasodilator-stimulated phosphoprotein
ROS	reactive oxygen species
sGC	soluble guanylate cyclase
shRNA	small hairpin RNA
SOD	superoxide dismutase
TAC	transverse aortic constriction
TBST	Tris-buffered saline/Tween-20
TGS	Tris/glycine/sodium dodecyl sulfide
TTC	2, 3, 5-triphenyl tetrazolium chloride
VASP	vasodilator-stimulated phosphoprotein

Abstract

PATHOGENIC ROLE OF PHOSPHODIESTERASE TYPE 5 UPREGULATION IN CARDIAC ISCHEMIA/REPERFUSION INJURY

By Daniel Christian Hobbs, B.S.

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

Virginia Commonwealth University, 2010

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Phosphodiesterase Type 5 **(**PDE5) inhibitors are cardioprotective against ischemia/reperfusion (I/R) injury. However, it remains uncertain if I/R affects PDE5. We hypothesized that generation of reactive oxygen species (ROS) during I/R leads to upregulation of PDE5, which contributes to pathological changes following acute myocardial infarction (AMI). Adult male ICR mice were subjected to 30 minutes of *in vivo* or *ex vivo* I/R. To examine the role of ROS, a subset of hearts were perfused with 100 μM hydrogen peroxide (H_2O_2). Expression and activity of PDE5, pPDE5, and cGMP-

dependent protein kinase (PKG) were measured by Western blots and spectrophotometric assay. The results show that ischemia and I/R significantly increased expression of PDE5. H_2O_2 had no effect on PDE5 expression and activity but significantly increased PKG activity. We conclude that acute cardiac ischemia or I/R upregulate PDE5 independent of oxidant stress in the heart.

Introduction

1.1 Coronary Heart Disease

Coronary heart disease (CHD) is the failure of the coronary circulation to supply adequate blood to the cardiac muscle and surrounding tissue. The American Heart Association has determined CHD, the cause of heart attacks and angina, will be the single leading cause of death in the United States this year just as it has been for the past sixty years, since 1950. Approximately 425,500 people a year now die of CHD which is nearly 75% of all deaths. In all, about every 25 seconds an American will suffer a coronary event, and about every minute someone will die from one such attack. In 2010, CHD will cost the United States $316.4 billion. This total includes the cost of health care services, medications, and lost productivity.[1] Taken together, these indicators constitute a rather bleak prognosis regarding the overall cardiovascular health of our country's population and indicate the urgency with which new medical therapies are needed in order to combat CHD and reduce its enormous national impact.

As previously mentioned, a heart attack, or acute myocardial infarction (AMI), is a severe condition caused by CHD. Every year about 785,000 Americans have a first AMI and 470,000 who have already had one or more will have another. All together AMI 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.[1] AMI occurs when there is a sudden occlusion of an artery supplying blood to cardiac muscle, or myocardium, depriving the area of blood and oxygen and causing dramatic changes in the cellular metabolism and function within this tissue. The occlusion leading to AMI is usually caused by a buildup of atheromatous plaques on the walls of the arteries that supply the myocardium. These plaques consist largely of macrophage cells containing cholesterol and fatty acids, calcium, and a variable amount of fibrous connective tissue. The resultant lack of blood flow and oxygen is called ischemia and if maintained for more than a few minutes, there is a loss of contracting myocardium in the affected area and a resultant increase in the workload on the remaining viable myocardium. This cardiac overload causes increased energy utilization and a 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.[2] Since the division of surrounding myocytes cannot replace those that have died from necrosis and apoptosis, preservation of the myocardium viability after ischemia has become a major target for therapeutic research.[3]

1.2 Ischemia/Reperfusion Injury

Following the occurrence of a detrimental occlusion of a coronary artery, it was shown in 1983 that reperfusion of the vessel was vital to protecting the ischemic myocardium and salvaging the remaining viable tissue.[4] Since that time, the advent of therapies such as thrombolysis, anti-platelet, and primary angioplasty have provided a means to reestablish coronary blood flow in the clinical setting. Yet paradoxically, the

reperfusion therapy itself, following ischemia, results in cardiomyocyte death; an inherent phenomenon termed ischemia/reperfusion (I/R) injury.

Cell death following I/R has been reported to have features of apoptosis, necrosis, and autophagy. Apoptosis, or programmed cell death, typically occurs via activation of caspases that cleave DNA and other cell components. Caspase 9 has been reported to be activated during ischemia, whereas caspases 8 and 9 are activated during reperfusion.[5] A number of studies have reported that addition of caspase inhibitors reduces infarct size, suggesting an important role for caspase activation in I/R injury.[5-7] Necrosis, or traumatic cell death, is characterized by cell swelling leading to irreversible rupture of the plasma membrane, release of cytosolic components, and finally an inflammatory response, which is an important component of I/R injury. The release of troponin and creatine kinase that occur during I/R are likely due to necrosis. A rise in cytosolic free Ca^{2+} as well as the production of reactive oxygen species (ROS) has been consistently observed in ischemia and early reperfusion. An increase in Ca^{2+} and ROS can lead to activation of an inner mitochondrial large-conductance channel known as the mitochondrial permeability transition pore (MPTP), causing loss of ATP and mitochondrial function. If a large number of mitochondria in a cell undergo MPTP, the cell will lose the capacity to make ATP and ion homeostasis, resulting in necrosis. Finally autophagy is a physiological mechanism that is used to remove damaged organelles, such as mitochondria or endoplasmic reticulum, and extensive autophagy can cause cell death. It has been shown that a decrease in a primary protein mediator of autophagy, beclin1, reduces I/R-mediated

autophagy and myocyte death.[8] Additionally, an increase in Ca^{2+}, which as stated occurs during ischemia, has been shown to increase autophagy.[9]

Ultimately, death following I/R injury appears to be a mixture of apoptotic, necrotic, and autophagy cell death, and it can have features of all three. The distinction between the modes of death may not be simple, since recent data suggest that all three forms can be regulated and are interrelated.[10,11] The primary issue is that cell death during I/R appears to be an active process, causing rapid disruption of heart tissue as shown by the release of certain cytosolic enzymes and changes in the microscopic structure of the myocytes. Although the restoration of blood flow proved to be crucial to reduce infarct size [4], the contradiction of reperfusion injury presents a clinical challenge in finding therapeutic strategies to limit and reduce I/R insult, thus preserving heart function.

1.3 Ischemic and Pharmacological Preconditioning

In 1986, a phenomenon called ischemic preconditioning (IP) was discovered involving brief episodes of ischemia applied to the myocardium that would consequently protect the tissue from subsequent, more prolonged periods of ischemia.[12] This incredibly innovative finding marked the beginning of a new field of research aimed at utilizing this phenomenon in the protection of the human heart from ischemic heart disease. The cardioprotective effects of IP have been shown to occur in two different phases or "windows". The early phase, referred to as "classic" preconditioning, is primarily mediated by activation of signaling pathways and posttranslational modification of proteins. It develops immediately after the IP maneuver and disappears within 1-2

hours.[13,14] The later phase, referred to as the "delayed" window of preconditioning, is largely mediated by gene induction and protein synthesis. It develops 12 hours later and lasts up to 96 hours.[15-18] Both phases of IP have been shown to reduce infarct size, contractile dysfunction, arrhythmias, generation of lactate, and rate of fall in ATP.[19-21]

Over the past 20 years, a great deal has been learned about the signaling pathways activated by IP and the cellular basis for its protection. The proposed IP mechanisms include the release of endogenous mediators (such as adenosine, norepinephrine, opioids, free radicals, and bradykinin),[22-26] activation of G protein-coupled receptors and the phosphatidylinositol 3-kinase (PI3K) pathway (and subsequent downstream targets such as protein kinase B [Akt], protein 70S6 kinase [p70S6K], protein kinase C [PKC], and extracellular signal-regulated kinase [ERK]),[27-31] opening of mitochondrial K_{ATP} channels,[32,33] and synthesis of cytoprotective proteins (including endothelial nitric oxide synthase [eNOS], inducible nitric oxide synthase [iNOS], cyclooxygenase-2, and heat shock protein 70).[21,34-36]

A number of pharmacological activators of IP mechanisms have been given exogenously to mimic the observed cardioprotective effects. These include adenosine receptor agonists, nitric oxide donors, bradykinin, anisomycin, cobalt chloride, and diazoxide; nevertheless, due to the lack of safe and effective drugs, none of the activators have been translated into clinical use.[37] Our laboratory has been at the forefront of this field of research and, over the course of the last 8 years, a pioneer in inventing the concept of pharmacological preconditioning with selective phosphodiesterase type 5 (PDE5) inhibitors. We have shown that drugs such as sildenafil (Viagra), vardenafil (Levitra) and

tadalafil (Cialis), which are known to enhance erectile function in men, also induce a powerful cardioprotective effect against I/R in rabbit and mouse hearts,[38,39] myocardial ischemia-induced heart failure,[2] and doxorubicin-induced cardiomyopathy in mice.[40] In a series of publications, our laboratory has shown that this preconditioning-like cardioprotective effect against I/R involved upregulation of eNOS and iNOS, activation of PKC/ERK, opening of mitochondrial K_{ATP} channels, and attenuation of apoptosis by increasing the B-Cell Lymphoma-2 (Bcl2)/ Bcl-2–associated X protein (bax) ratio.[38,39,41-43] Still, as diverse as these mechanisms are, none has shown a direct involvement of PDE5 or relate to the known pharmacological action of sildenafil on inhibition of PDE5.

1.4 Phosphodiesterase Type 5

The intracellular second messengers cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) both serve vital roles in cardiovascular regulation by influencing function, gene expression, and morphology. cAMP is a mediator of many effects of epinephrine on the heart and other tissues while cGMP mediates nitric oxide (NO) and natriuretic peptide (NP) action.[44-53] In 1958 cAMP was first discovered and this report was soon followed by that of cGMP in 1963.[54,55] The search was then on for researchers to quickly identify enzymes that specifically hydrolyzed these messengers. Eventually, 11 different 3',5'-cyclic nucleotide phosphodiesterase (PDE) isoenzyme families (PDE1-PDE11) were identified and grouped according to their ability to hydrolyze cAMP and cGMP to the linear 5'-nucleoside monophosphates AMP and GMP, respectively. This reaction is highly exothermic (> 10 kcal/mol), making it

essentially irreversible in the cell.[56,57] Thus, by producing inert molecules and destroying the second messenger activity of cyclic nucleotides, PDEs play critical roles in a wide range of physiological and pathological processes.

PDE5 was originally characterized in the context of an unidentified binding protein for cGMP and not as a PDE.[58] Early descriptions noted a high-affinity cGMP-binding protein present that did not comigrate with cGMP-dependent protein kinase (PKG) activity, and at that time PKG was the only known high-affinity binding protein for cGMP. Within a few years, PDE5 was identified, isolated, and characterized from platelets and later lung.[59-61] It was discovered to be functionally specific for cGMP hydrolysis along with the presence of high affinity-binding sites for cGMP. At the time, it was initially thought that PDE5 might be related to the already well understood, light sensitive, cGMP-selective PDE found in the eye, which is now known as phosphodiesterase type 6 (PDE6). Both enzyme families selectively used cGMP compared with cAMP as substrate, both bound cGMP with high affinity, both were inhibited by many of the same agents, and both had a very similar size. However, differences in V_{max} activities and the lack of regulation of PDE5 by light suggested that they might be different PDEs. So there existed a continuing confusion in the scientific literature regarding the molecular identity of the cGMP-specific PDEs and PDE5 was originally labeled as the "cGMP-binding, cGMP-specific PDE." The issue was finally resolved when the bovine lung cGMP-binding PDE was cloned, expressed, and sequenced.[62] It was only upon comparison with the previously published PDE6 sequences that it was entirely clear PDE5 was a distinct gene product.[63]

Presently, PDE5 is now known to be dimeric with each monomer approximately 90 to 100 kD in size, depending on species. The carboxyl terminal portion contains the hydrolyzing catalytic domain of ~230 amino acids, encompassing two zinc-binding motifs and a catalytic cGMP binding site. The amino terminal portion contains two allosteric cGMP binding sites of ~90 amino acids each and at least a portion of the dimerization domain. These N-terminus binding sites are today called regulatory GAF domains of the enzyme, an acronym derived from the names of the first three classes of proteins recognized to contain these sequences, i.e., cGMP-binding PDEs, *Anabaena* adenylyl cyclase, and *Escherichia coli* Fh1A.[64] The two GAF domains (GAF-A and GAF-B) are highly homologous, but cGMP binding occurs only to the GAF-A domain (K_D < 40 nM) of PDE5.[65] Cyclic nucleotide binding to this domain is greater than 100-fold selective for cGMP over cAMP, [65] and it has been shown that the allosteric binding of cGMP to the GAF-A domain stimulates enzyme catalytic activity about 10-fold, while blockage of this allosteric binding causes inhibited activity.[66] Moreover, the affinity of this site for cGMP is increased by phosphorylation of nearby serine 92, which is just adjacent to the GAF-A domain, via PKG thereby stabilizing the activated form of PDE5.[67,68] Since the original cloning of the PDE5 cDNA, only one PDE5 gene has been discovered to date, PDE5A, although 3 variants of the PDE5A mRNA and protein have been identified under the control of differentially regulated promoters.[69-71] PDE5A1, PDE5A2, and PDE5A3 differ at their N-terminal regions, and all 3 have unique first exons followed by a common sequence of 823 amino acids. While it has not been proven, it is postulated that the different promoters for the PDE5 isoforms allow differential control of PDE5 gene

expression and provide a mechanism for longer term regulation than just the allosteric binding or phosphorylation previously mentioned.

Interestingly, despite it being one of the first of the 11 PDE families found to be cGMP specific, PDE5 received little notoriety or attention until studies discovered its role as a regulator of vascular smooth muscle tone.[72-74] By the mid 1980's, pharmacologists at Pfizer research laboratories were working on an antihypertension drug and postulated that a selective cGMP PDE inhibitor might relax arteries and lower blood pressure by augmenting intracellular cGMP. In screening for agents that inhibited cGMP hydrolysis but not cAMP, they found a cGMP PDE selective agent, UK-92 480, which was first targeted clinically at angina pectoris. The tests proved to be disappointing, with very little cardiovascular impact, however patients reported "side effects" at high doses, which included penile erections. Pfizer shifted its indication to treatment for erectile dysfunction and sildenafil (Viagra) was created in 1998 as the first isozyme-selective PDE inhibitor in wide clinical use. This "urological detour" away from investigating direct cardiovascular applications of PDE5 inhibition helps to explain why it took considerable time before focus was diligently applied to PDE5 with respect to cardiovascular disease and regulation.

1.5 PDE5 and Cardiac Function

The primary effect of PDE5 activity is to hydrolyze cGMP and thus lower its intracellular levels. In turn, cGMP can influence other PDEs responsible for regulating cAMP by binding to their allosteric or catalytic sites. Phosphodiesterase type 2 (PDE2) is expressed in myocytes and has regulatory GAF domains that can bind cGMP and in turn

enhance cAMP hydrolysis.[75,76] Alternatively, cGMP can competitively bind to the catalytic site of phosphodiesterase type 3 (PDE3). This enzyme binds both cAMP and cGMP at high affinity and similar K_m but has a V_{max} for cAMP 10-fold greater, conferring catalytic specificity. At higher levels cGMP may inhibit PDE3 hydrolysis, resulting in enhanced cAMP which influences cardiac rhythm (chronotropy), contractile force (inotropy) and relaxation (lusitropy) while additionally playing a role in platelet aggregation and endothelial cell permeability (Fig. 2).[77-81]

Still, of vital importance is cGMP's ability to stimulate PKG and this is presently thought to be the major effect of PDE5 activity on vessels and the heart. As previously mentioned, the classical role of PDE5 is in modulation of vascular smooth muscle contraction. In this case, cGMP is generated either from soluble guanylate cyclase (sGC) present in the cytosol (triggered by NO) or from particulate guanylate cyclase (pGC) present at the plasma membrane (triggerd by NP). The generated cGMP activates PKG which then via several mechanisms causes muscle relaxation. These mechanisms involve decreasing intracellular free calcium concentrations,[82,83] reducing calcium sensitization,[84-86] and regulating thin filament proteins (Fig. 1).[87-94] Thus PDE5 functions, by catalyzing the hydrolysis of cGMP, to antagonize all of these observed effects and intriguing emerging evidence is beginning to show this role may be more significant in cardiac function than originally considered.

The presence of PDE5 within the heart has been a contentious issue with many positive and negative findings.[95-100] Our laboratory was able to detect PDE5 gene and protein expression in the mouse heart and cardiomyocytes by RT-PCR, immunofluorescent

staining, and Western blotting.[43] Still, despite this discovery combined with other early evidence of myocardial PDE5 gene expression,[69,101] its protein synthesis and enzyme activity are rather low compared with other tissues such as lung, and were traditionally thought to be physiologically insignificant.[99,102] In spite of this, more recent studies have reported evidence of both detectable PDE5 mRNA and protein expression in isolated cardiomyocytes, as well as the physiologic effects of PDE5 inhibition in myocytes and whole heart.[43,96,97,103] There is also growing evidence indicating the importance of compartmentation in cyclic nucleotide PDE modulation.[104] Myocytes transfected with a cGMP-sensitive Ca^{2+} reporter channel recorded increased membrane cGMP induced by NP stimulation that was completely unchanged by concomitant PDE5 inhibition. This contrasted to the signal generated by NO donors, which sildenafil enhanced.[105] Therefore, it seems that PDE5 exerts a specific spatiotemporal control on the pool of intracellular cGMP synthesized by sGC but not that generated by pGC. Additionally, although the levels of PDE5 in cardiomyocytes are rather low, newer data suggest its regulatory effects may be due to it being primarily localized to the Z-line in healthy animals, while under pathological conditions it is dispersed throughout the cell.[43,96,103,106]

The level of PDE5 within the heart appears to be influenced by the occurrence of heart disease and it confers targeted regulation of stress responses despite having little influence on basal function. PDE5 expression is reportedly increased in human and animal models of right ventricular hypertrophy.[107] In these studies, PDE5 inhibition significantly increased contractility, cGMP, and cAMP in hypertrophied right ventricles but not in normal right ventricles. Studies have also shown that cGMP catabolism by PDE5 is

increased in chamber and myocyte hypertrophy which can be suppressed by the administration of PDE5 inhibitors.[108] Increased PDE5 expression has also been shown in left ventricles of humans with end-stage ischemic and dilated cardiomyopathy. [109,110] These studies additionally demonstrated that a cardiomyocyte-specific overexpression of PDE5 in transgenic mice predominately localized to Z-bands and predisposed them to adverse left ventricle remodeling following AMI. Along these same lines, it has been reported that myocyte hypertrophy is blunted by PDE5 gene silencing with small hairpin RNA (shRNA).[106] PDE5 upregulation has also been cited in a number of other chronic cardiovascular diseases including pulmonary hypertension and congestive heart failure.[111,112] Still, pathological regulation over PDE5 is scantily dealt with in the existing literature and its behavior has not been studied in more acute models of heart disease like I/R.

1.6 PDE5 and Reactive Oxygen Species

Aerobic organisms create energy needed to fuel biological functions by oxidative phosphorylation, a process which involves the transport of hydrogen ions across the inner mitochondrial membrane by means of the electron transport chain. In the electron transport chain, electrons are passed through a series of proteins via redox reactions. The last destination for an electron along this chain is an oxygen molecule. Normally the oxygen is reduced to produce water, but sometimes oxygen is instead prematurely and incompletely reduced to create reactive oxygen species (ROS) such as superoxide radicals ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2).[113-117] This "physiologic" generation of ROS is

normally inactivated by endogenous antioxidant scavenger mechanisms present within the cells.[118]

During ischemia, ROS are generated within and can lead to damage to the electron transport chain of mitochondria.[119-121] Upon reperfusion, with the return of oxygen, a large burst of ROS is seen.[122-124] This increase in ROS during ischemia and reperfusion is thought to be due to damage to electron transport chain components resulting in inefficient transfer of electrons. This toxic ROS overproduction can damage or deplete endogenous antioxidants and possibly contribute to irreversible damage of mitochondrial function and consequently impaired recovery of physiological function and cell death.[125-130]

Recent studies have led to consideration of a correlation between ROS and PDE5 expression/activity. It has been demonstrated that exposing fetal pulmonary artery smooth muscle cells (FPASMCs) to hyperoxia leads to a decreased intracellular cGMP response, increased PDE5 phosphorylation, mRNA and protein expression, as well as increased cGMP hydrolytic activity. Treatment of normoxic FPASMCs with exogenous H_2O_2 induced PDE5 expression and activity, suggesting that ROS potentially mediate the PDE5-enhancing effects of hyperoxia in FPASMCs. Additionally, treatment with a chemical antioxidant, N-acetyl-cysteine, was sufficient to block the increases in PDE5 expression and activity.[131] An extremely current work has shown that in mice, following transverse aortic constriction (TAC)-induced congestive heart failure (CHF), an increase of myocardial PDE5 expression positively correlated with the levels of oxidative stress markers 3'-nitrotyrosine and 4-hydroxynonenal expression. The superoxide dismutase (SOD) mimetic M40401 attenuated oxidative stress and blunted PDE5 expression and

activity as well as CHF, while increased oxidative stress in SOD3 knockout mice exacerbated the increase in PDE5 expression and CHF following TAC.[110] These studies are intriguing and lend credence to the possibility that oxidative stress could regulate PDE5 within the heart.

1.7 Rationale of the Present Study

The purpose of the current investigation was to identify the role of PDE5 within the heart and specifically its response to acute cardiac I/R injury. We hypothesized that the detrimental events of ischemia and reperfusion lead to a deleterious upregulation of PDE5, which is connected to the overproduction of ROS and contributes to the adverse cardiac stress response and pathological changes following AMI. Of particular interest was defining PDE5 within the signaling mechanisms underlying the already proven cardioproctective action of selective PDE5 inhibitors.

To test this hypothesis we used a murine model of *in vivo* regional I/R where we occluded the left anterior descending coronary artery (LAD) for 30 minutes and reperfused the heart for varying durations of time. We additionally used a Langendorff isolated perfused mouse heart model to administer 30 minutes of global ischemia and varying durations of reperfusion. This *ex vivo* model also allowed us to examine the effects of exogenous oxidant stress upon the heart via perfusion with the oxygen radical generator H_2O_2.

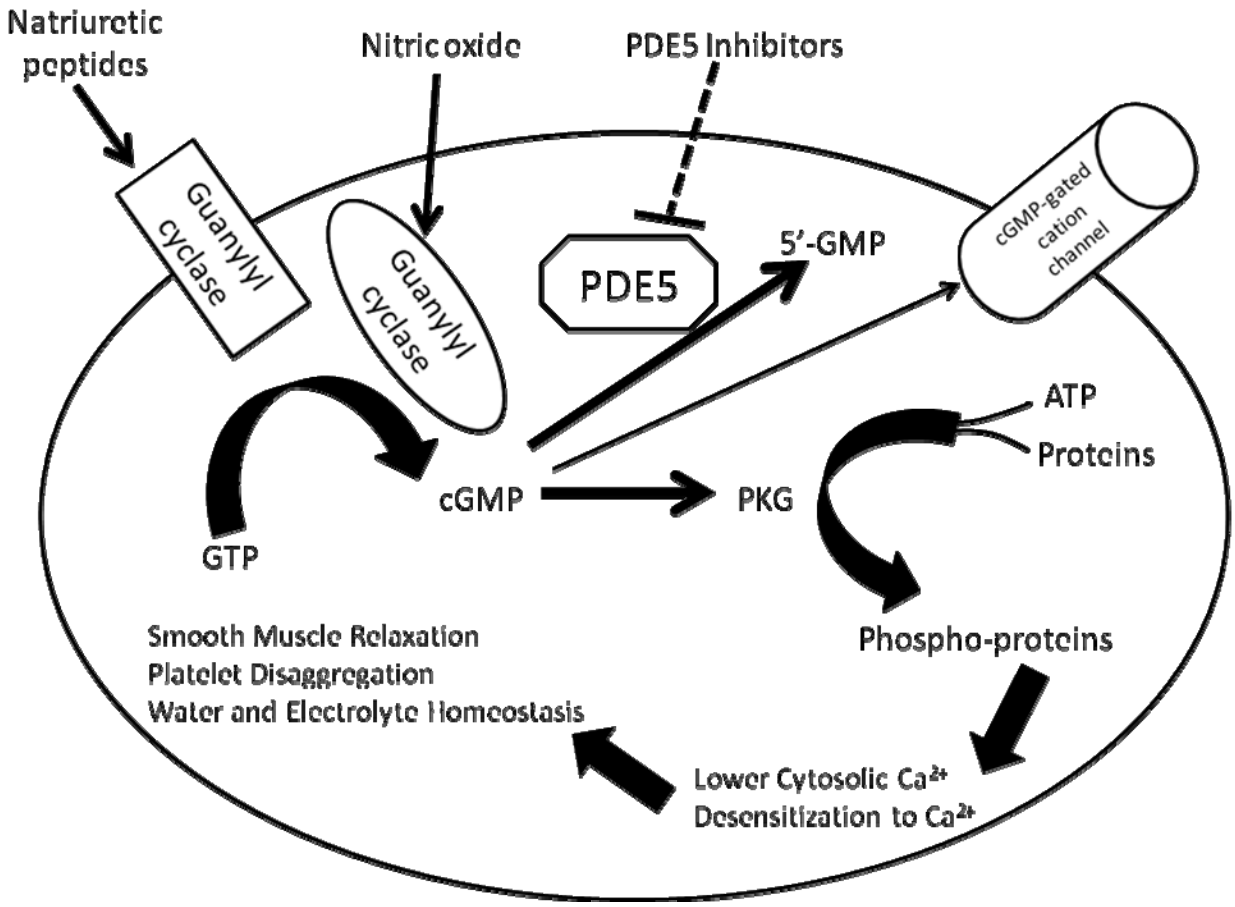


Figure 1: General scheme for the role of PDE5 in cGMP-signaling pathways.

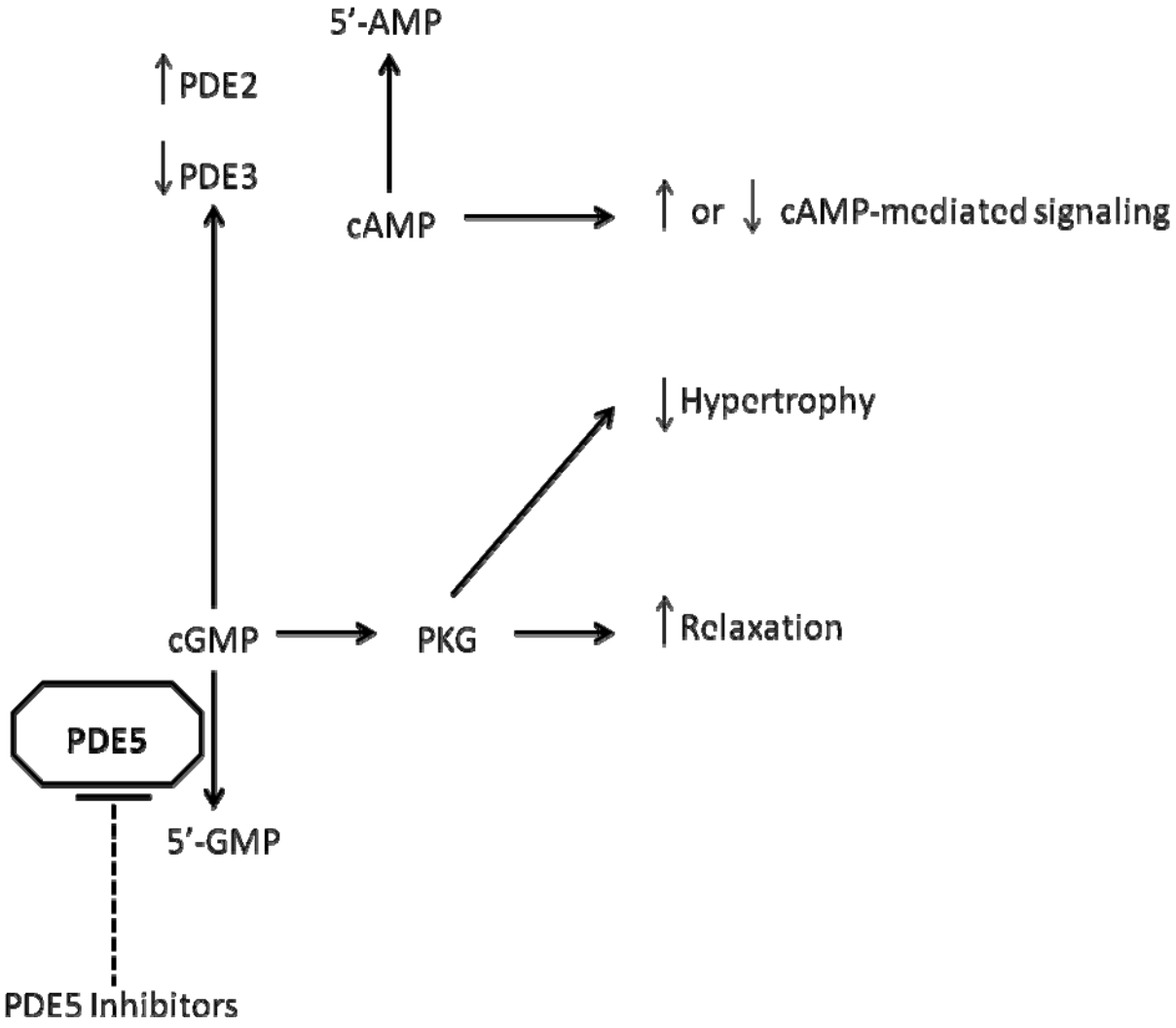


Figure 2: Overview of PDE5 in cardiac muscle.
Inhibition of PDE5 leads to a rise in intracellular cGMP and activation of PKG. This may result in muscle relaxation and antihypertrophic effects. The rise in cGMP may potentiate cAMP-mediated signaling in cardiac muscle by inhibition of PDE3 or reduce cAMP-mediated signaling by stimulation of PDE2

Materials and Methods

2.1 Animals

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

2.2 Drugs and chemicals

Unless otherwise specified, all the chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Chemicals include those for preparing the K-H buffer in Langendorff isolated heart experiments (i.e. NaCl, $NaHCO_3$, $CaCl_2$, KCL, KH_2PO_4, $MgSO_4$, glucose, EDTA) as well as H_2O_2. Sildenafil is a pure solid powder and was kindly provided by Pfizer, Inc. (New York, NY) and dissolved in water. Pentobarbital sodium was the anesthetic used for all the surgeries and was supplied by Ovation Pharmaceuticals, Inc. (Deerfield, IL).

2.3 Myocardial Infarction

Prior to surgery, adult ICR mice were weighed and anesthetized with pentobarbital sodium (70 mg/kg, ip). They were intubated orotracheally and ventilated on a positive-pressure rodent ventilator (Harvard apparatus), set at 133 cycles/min and a tidal volume of 0.2 ml. A left thoracotomy was performed at the fourth intercostal space in order to expose the heart. Upon visualizing the LAD under a magnifier or microscope, a 7.0 silk ligature was passed underneath the artery and a piece of polyethylene (PE)-10 tubing was placed on the artery. Ischemia was induced by tying the PE-10 tubing onto the artery with the ligature. The tubing was removed after 30 minutes of ischemia in order to reperfuse the vessel and the silk ligature was left in place to mark the risk area for ischemia. A 5.0 silk suture was used to close the chest. The animals were extubated and injected intramuscularly with analgesic (Buprenex; 0.02 mg/kg) for pain reduction, and antibiotic (gentamicin; 0.7 mg/kg) to prevent infection. Afterward, the animals were allowed to recover. Following the desired length of reperfusion each mouse was again anesthetized with pentobarbital sodium (100 mg/kg, ip). The chest was then opened at the sternum, and the heart was rapidly removed from the thorax and harvested at the different time points (Fig. 3). The hearts were rinsed in PBS to remove blood, snap frozen in liquid nitrogen, and immediately stored in -80°C conditions until further use for biochemical/molecular assays.

2.4 Measurement of infarct size

Results were obtained from my lab group involving the use of PDE5 inhibitors and their effect on the subsequent infarct size following ligation. Directly relevant to this project

was the protocol in which adult ICR mice received an injection of saline (0.2 mL, ip) or sildenafil (0.71 mg/kg in 0.2 mL saline, ip) 1 hour before undergoing 30 minutes of ischemia followed by 24 hours of reperfusion (Fig. 4). At the end of each experiment, the hearts were washed of blood, and ~2 mL of 10% Evans blue dye was injected as a bolus into the aorta until most of the heart turned blue. The heart was then washed with saline to remove the excess Evans blue. Finally, the heart was frozen at -20°C. The frozen hearts were then cut by hand using a surgical blade parallel to the atrioventricular groove, into 6 to 8 transverse slices of approximately equal thickness (~1mm). The heart slices were incubated in a 10% 2, 3, 5-triphenyl tetrazolium chloride (TTC, Sigma) solution for 30 minutes at room temperature. In normal myocardium, TTC is converted by dehydrogenase enzyme to a dark red formazan pigment. On the other hand, infracted myocardium, due to loss of dehydrogenase enzyme following cell membrane rupture, does not take up TTC stain and remains a pale color. After staining, the TTC was removed and replaced with 10% formaldehyde for additional 2-4 hours to fix the heart slices. Thereafter, the heart slices were digitally imaged and the areas of infarcted tissue, the risk zone, and the whole left ventricle were measured using computer morphometry (Bioquant98). 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 (Fig. 5).

2.5 Langendorff isolated heart preparation

Prior to surgery, adult ICR mice were weighed and anesthetized deeply with pentobarbital sodium. Anesthesia was prepared by mixing 0.2 mL of heparin to protect the heart against

microthrombi, 0.4mL of pentobarbital, and 0.2 mL saline. Each mouse was injected intraperitoneally with 0.2 mL of anesthesia (100 mg/kg). The chest was then opened at the sternum, and the heart was rapidly removed from the thorax and placed in a small dish containing ice-cold Krebs-Henseleit (K-H) buffer and heparin (0.1 mL per dish), where it was trimmed of excessive tissue and fat. The aortic opening was exposed and quickly cannulated on a 20-gauge blunt needle and double loop tied using a 5.0 silk suture. The heart was then retrogradely perfused at a constant pressure of 55 mmHg through the aorta in a non-recirculating Langendorff apparatus (Fig. 6) with modified K-H buffer [containing (in mM) 118 NaCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 4.7 KCL, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, and 0.5 EDTA]. The K-H solution was pre-filtered by a micro-filter (Millipore Corp.) with a pore size of 0.45 μm diameter, and was continuously gassed with 95% O_2 + 5% CO_2 (pH ~ 7.4). The perfusion solution was warmed to 40 ºC through a water-jacketed glass cylinder/heat exchanger system with a warming/cooling bath and automatic thermal controller (Brinkmann) and was constantly circulated by a varistaltic water pump (Manostat). The solution was warmed to 40 ºC to compensate for any heat loss experienced as the buffer circulates through the Langendorff apparatus. The ambient temperature around the heart was also kept at 37 ± 0.2 ºC throughout the experiment using a 100 Watt heating lamp. A digital thermometer (VWR) continuously monitored the air temperature around the heart. Hearts were retrogradely perfused for the control, ischemia alone, or ischemia plus reperfusion groups at the different time points shown (Fig. 7). For ischemia and reperfusion, following the 30 minute stabilization period, the hearts were subjected to a 30 minute no-flow normothermic global ischemia by closing off a stopcock

connected to the aortic cannula and reperfusion was started by reopening the stopcock of the aortic cannula and continued for 30 minutes. In separate experiments, H_2O_2 (100 μM) was administered for 10 or 30 minutes via a syringe pump to the heart in conjunction with K-H buffer (Fig. 8). Following 20 and 30 minute stabilization periods, respectively, the hearts were subjected to H_2O_2 perfusion by opening a side-port of the stopcock connected to the aortic cannula. Once the appropriate time (10 or 30 minutes) of infusion had been achieved the stopcock was switched back to perfusion solely with K-H buffer for 30 minutes. Following treatment in both experiments, the hearts were then cut off of the Langendorff apparatus removing the atria, and snap frozen in liquid nitrogen and immediately stored in -80°C conditions until further use.

2.6 Protein extraction

Adult ICR mice hearts were snap frozen in liquid nitrogen and immediately stored at –80°C following both myocardial infarction and Langendorff isolated heart protocols. Each heart sample was crushed using a mortar and pestle. Liquid nitrogen was added as needed during this process to prevent the tissue sample from thawing. Once the sample was crushed into a fine powder, 1.0 mL of ice-cold lysis buffer [containing (in mM) 20 Tris* HCL pH 7.4, 150 NaCl, 1 EDTA, 1 EGTA, 0.2 PMSF, 0.2 Na_3VO_4, and 5 β-Mercaptoethanol, with protease inhibitor cocktail (Pierce) and phosphatase inhibitor cocktail (Pierce) (10 μL of each inhibitor per mL of buffer used)] was added to each sample tube. The samples were then homogenized with a Glas-Col® Tissue Homogenizing System, transferred to Eppendorff tubes, and sonicated with a Heat Systems Inc. Sonicator

Ultrasonic Processor XL. Each sample was kept on ice for an additional 30 minutes before being centrifuged at 12,000 G (Eppendorf Centrifuge 5810R) at 4°C for 15 minutes. The supernatant was separated from the pellet, divided up into 3 aliquots, purified over Zeba Desalting Spin Columns (Thermo Fisher Scientific, Rockford, IL) and stored in –80°C conditions for further analysis. Protein content of each heart sample was determined by using 10 mg of bovine serum albumin (BSA, Sigma-Aldrich) in 10mL of distilled water, forming a BSA stock solution. Taking 1 mL of the solution and adding it to 19 mL of distilled water diluted this stock solution. This second solution was used to establish a standard curve by adding different amounts of the BSA solution, water and protein assay dye (Bio-Rad Laboratories, Inc., Hercules, CA). To determine the protein concentration of our collected samples, 2 µl of each heart tissue sample were added to 798 µl of water along with 200 µl of protein assay dye before protein concentration was determined by performing the Bradford assay using a SmartSpecTM 3000 spectrophotometer (Bio-Rad).[132]

2.7 Western Blot

Protein samples were combined with an equal volume of a loading buffer solution made by mixing 25 µl of Laemmli sample buffer (Bio-Rad), and 475 µl β-mercaptoethanol (Bio-Rad). The samples were boiled for 5 minutes and centrifuged for 20 seconds at 14,000 rpm at room temperature before being loaded into each well on polyacrylamide gels (Bio-Rad). A protein marker (Bio-Rad) was also used for better identifying the molecular weight of the target protein. The gels were electrophoresed by a Bio-Rad energy pack for

1 hour at 180 volts using a running buffer [1X Tris/glycine/sodium dodecyl sulfide (TGS)]. These electrophoresed proteins were trans-blotted from the gel to nitrocellulose membranes (Bio-Rad) for 90 minutes at 400 mA in 4°C using a transfer buffer (700 mL water, 200 mL methanol, 100 mL 10X TGS). After transfer, the nitrocellulose membranes were placed for 1 hour in blocking solution [5% nonfat dry milk (Bio-Rad), in 1X Tris-buffered saline (Bio-Rad) and 0.05% Tween-20 (Bio-Rad) wash (1X TBST)] at room temperature to inhibit nonspecific binding. Subsequently, the membranes were incubated with primary antibodies on a rocking platform overnight in a 4°C cold room at the appropriate dilution in 5% milk in 1XTBST (1:500 for PDE5A [rabbit polyclonal] [Santa Cruz Biotechnology, Inc., Santa Cruz, CA], 1:1000 for α-Tubulin [goat polyclonal] [Santa Cruz], 1:500 for phosphorylated-vasodilator-stimulated phosphoprotein [pVASP][Ser239] [rabbit polyclonal] [Cell Signaling Technology, Inc., Boston, MA], 1:500 for phosphorylated-phosphodiesterase type 5 [pPDE5][Ser92] [rabbit polyclonal] [Fabgennix, Inc., Frisco, TX], and 1:1000 for vasodilator-stimulated phosphoprotein [VASP] [rabbit polyclonal] [Cell Signaling]). After being washed, the blots were incubated with anti-rabbit (GE Healthcare, Buckinghamshire, UK) or anti-goat (Santa Cruz) secondary antibody conjugated to horseradish peroxidase at a 1:2000 dilution in 5% milk in 1XTBST on a rocking platform for 1 hour at room temperature. Membranes were then washed, and the bands were visualized via enhanced chemiluminescence (PerkinElmer, Waltham, MA) and film in a dark room which was subsequently developed by a film processor (Kodak, Rochester, NY). The bands of proteins were quantified using the Image J (Image Processing and Analysis in Java) computer program. Expression for PDE5 and pPDE5

were normalized to α-Tubulin expression while expression for pVASP was normalized to VASP expression.

2.8 Activity Assay

Protein samples were assayed for cGMP hydrolytic activity using a commercially available spectrophotometric cyclic nucleotide phosphodiesterase assay kit (Biomol, Plymouth Meeting, PA). The basis for the assay is the cleavage of cGMP by a cyclic nucleotide phosphodiesterase, such as PDE5, followed by the release of free phosphate by a 5'-nucleotidase from *Crotalus atrox* venom. The free phosphate is quantified using the Biomol Green reagent (Biomol) in a modified Malachite Green assay.[133,134] The reactions were mixed in a 96-well plate, and started in a timed fashion by addition of phosphodiesterase. Each sample was read in four wells, two without sildenafil and two with sildenafil (250 nM), to determine PDE5 specific cGMP hydrolytic activity. The samples were then incubated at 30°C for 30 minutes and stopped in a timed fashion by addition of the Biomol Green reagent (Biomol). The samples were incubated with the Biomol Green reagent at room temperature for 20 minutes. Results were measured using a SpectraMax Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) at 620 nm wavelength and compared to a standard curve generated with GMP and 5'-nucleotidase. The difference between the pmol cGMP hydrolyzed per µg total protein per minute without sildenafil and the pmol cGMP hydrolyzed per µg total protein per minute with sildenafil represents the PDE5 specific cGMP hydrolytic activity. Results are shown

as the PDE5 specific pmol cGMP hydrolyzed per µg total protein per minute for each sample.

2.9 Cytotoxicity Assay

In the Langendorff isolated heart preparations, the leakage of lactate dehydrogenase (LDH) into the isolated mouse heart coronary effluent was assayed spectrophotometrically using a commercially available kit (BioVision, Mountain View, CA). The intracellular enzyme LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released upon damage to the plasma membrane. The basis for the assay is a coupled enzymatic reaction whereby LDH oxidizes lactate to pyruvate which then reacts with tetrazolium salt to form formazan which can be quantified by a formazan dye. Thus the increase in the amount of formazan produced directly correlates to the increase in the number of lysed cells and functions as a way of monitoring cardiomyocyte death. The coronary effluent was collected from the hearts at 1 minute prior to the global ischemia or H_2O_2 as well as 5, 10, 20, and 30 minutes during the reperfusion period (Fig. 7 & 8). The reactions were mixed in a 96-well plate, and started in a timed fashion by addition of 100µL of reaction mixture to 100µL of coronary effluent. Each sample was prepared in duplicate and incubated at room temperature for 30 minutes while being protected from light. Results were measured using a SpectraMax Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) at 490 nm wavelength and normalized against the individual coronary flow rate.

2.10 Data analysis and statistics

For each experiment, data from samples were calculated and expressed as means ± SEM. The significance of differences between experimental conditions was determined by unpaired t-tests. $P < 0.05$ was considered to be statistically significant.

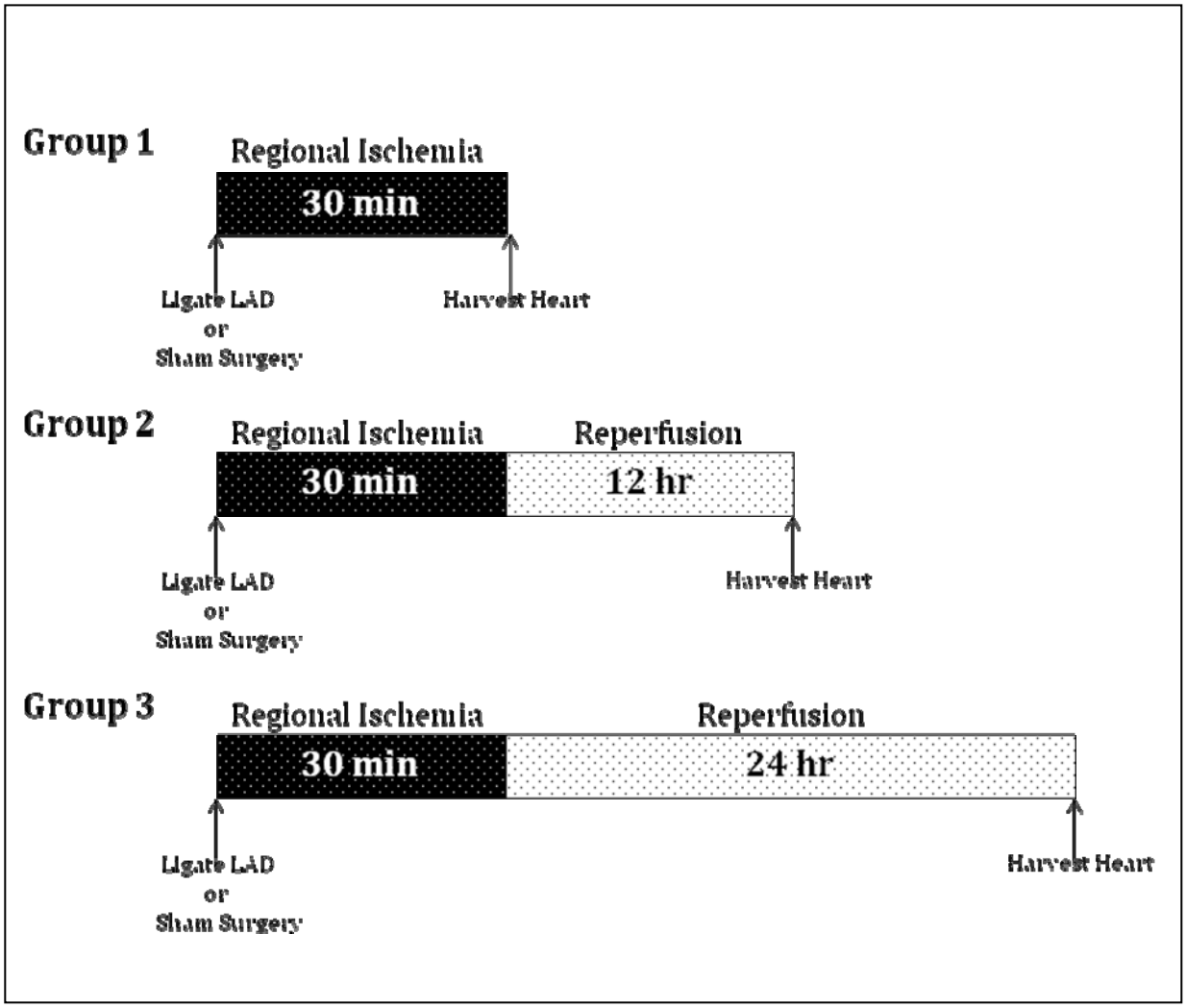


Figure 3: Myocardial infarction protocol for ischemia and I/R.
Experimental protocol for studies of PDE5 following Ischemia and I/R. Arrows indicate the time points for performance of surgical procedures.

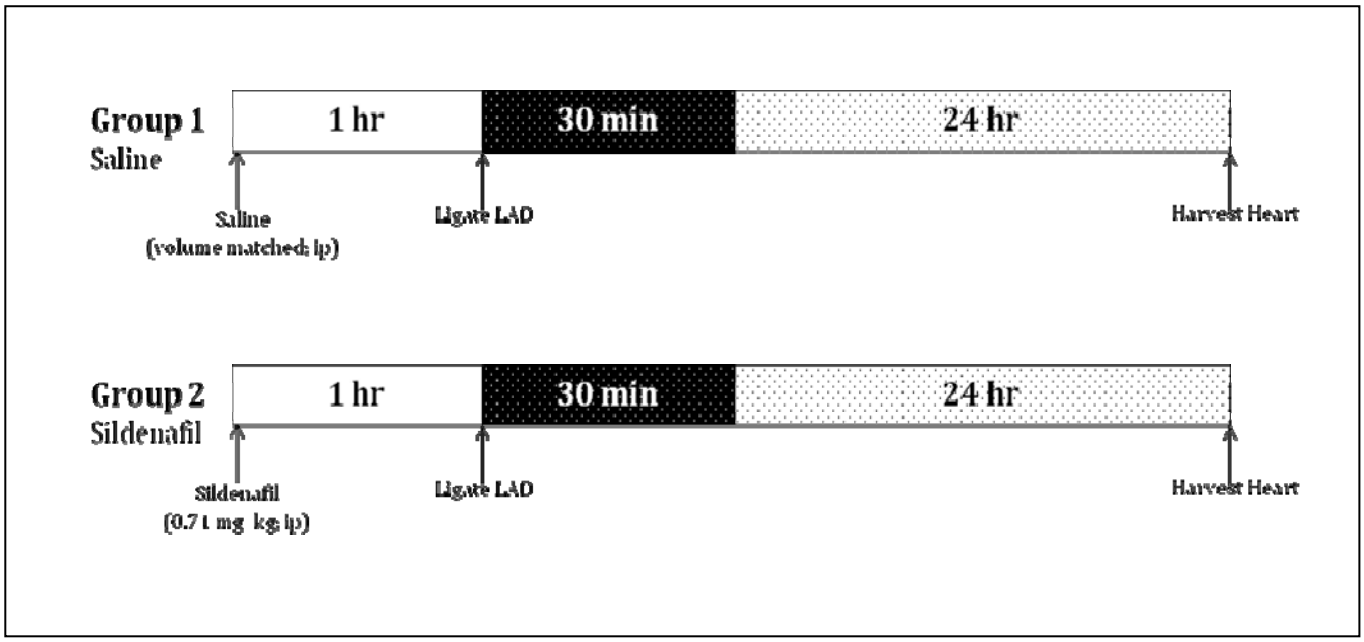


Figure 4: Protocol for studies of sildenafil therapy on myocardial infarction. Experimental protocol for sildenafil administration prior to myocardial infarction. Sildenafil (0.71 mg/kg) was administered intraperitoneally 1 hour before left anterior descending coronary aretery (LAD) ligation. Arrows indicate the time points for saline/sildenafil treatment and performance of surgical procedures.

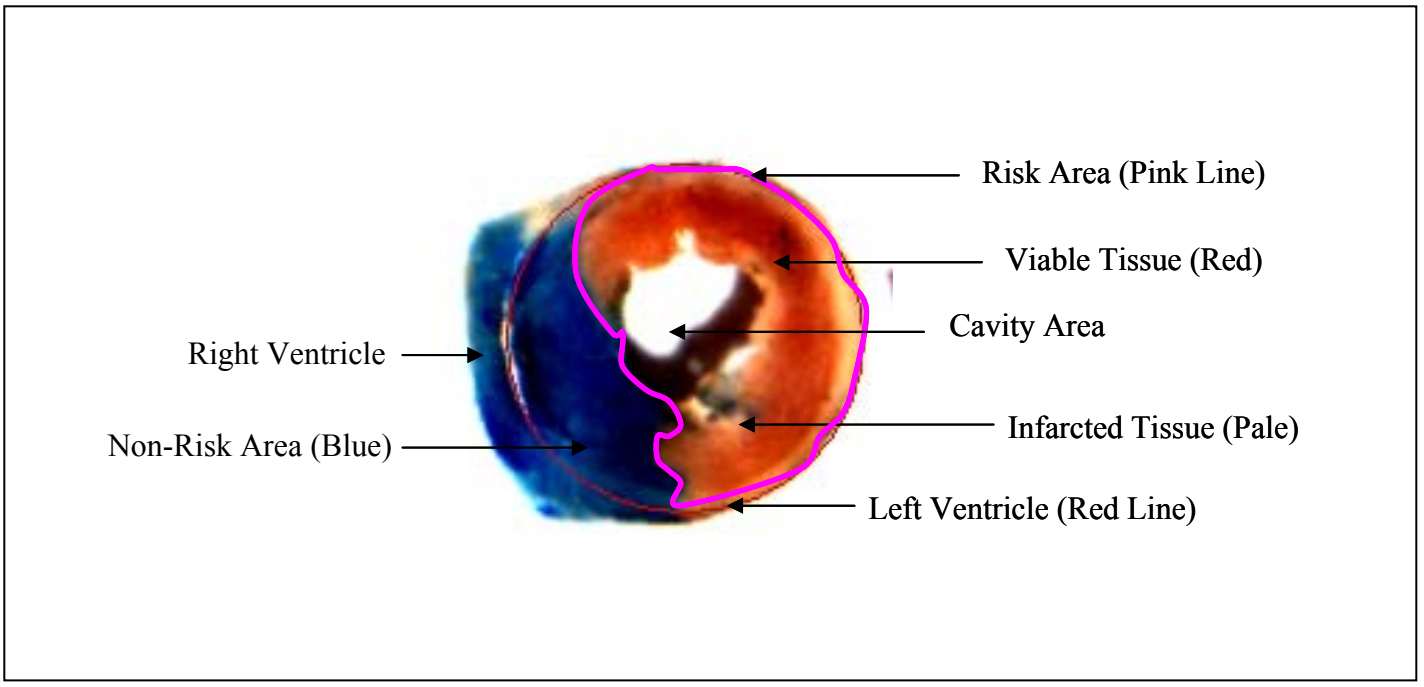


Figure 5: Representative section of mouse heart.
This cross section shows the criteria used to determine the risk area, non-risk area, infracted tissue, cavity area, and viable tissue. The risk area was calculated as total ventricular area minus the area of the cavities. The infarct size was calculated as a percentage of the risk area.

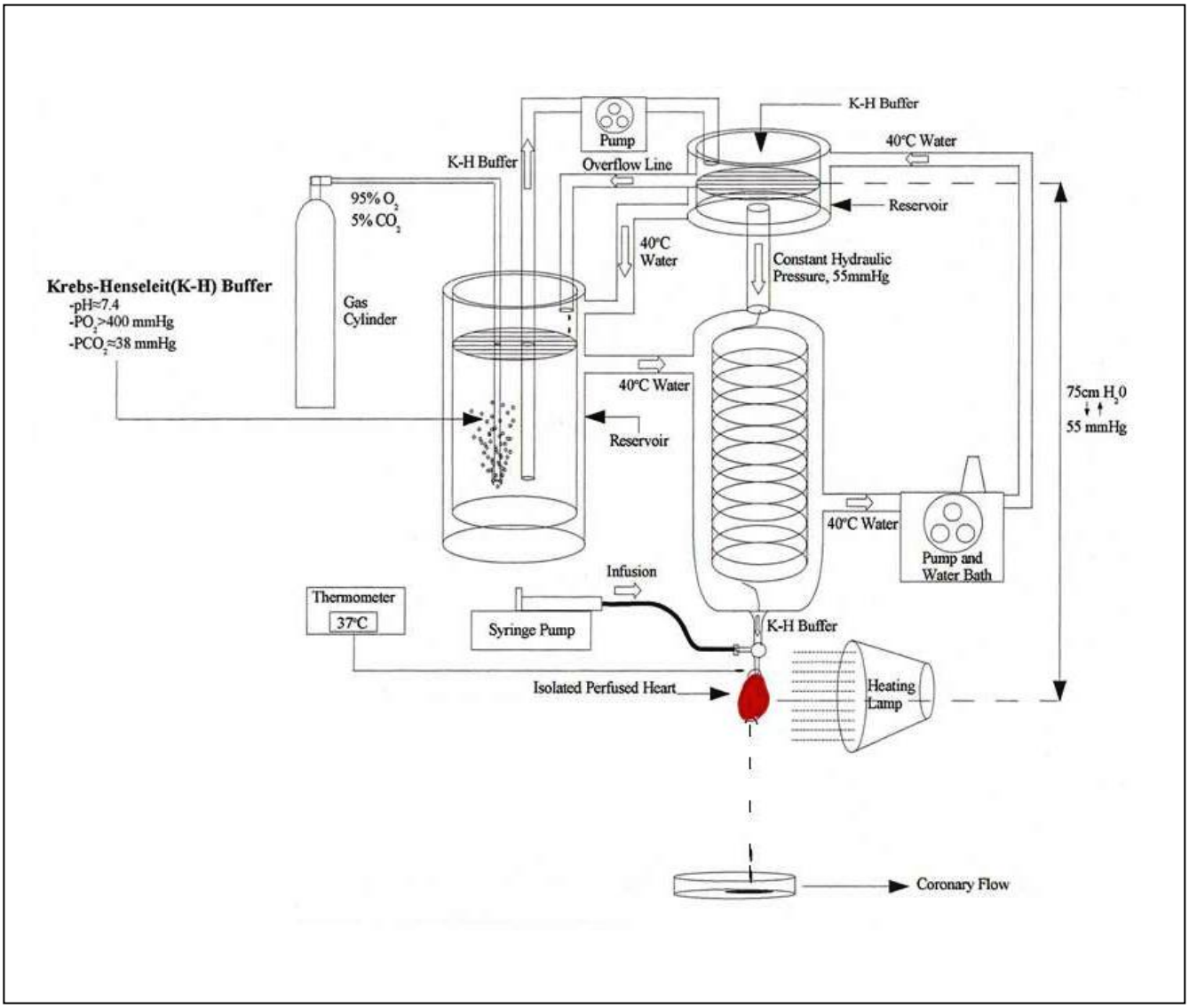


Figure 6: Illustrative description of the Langendorff isolated perfused heart system.

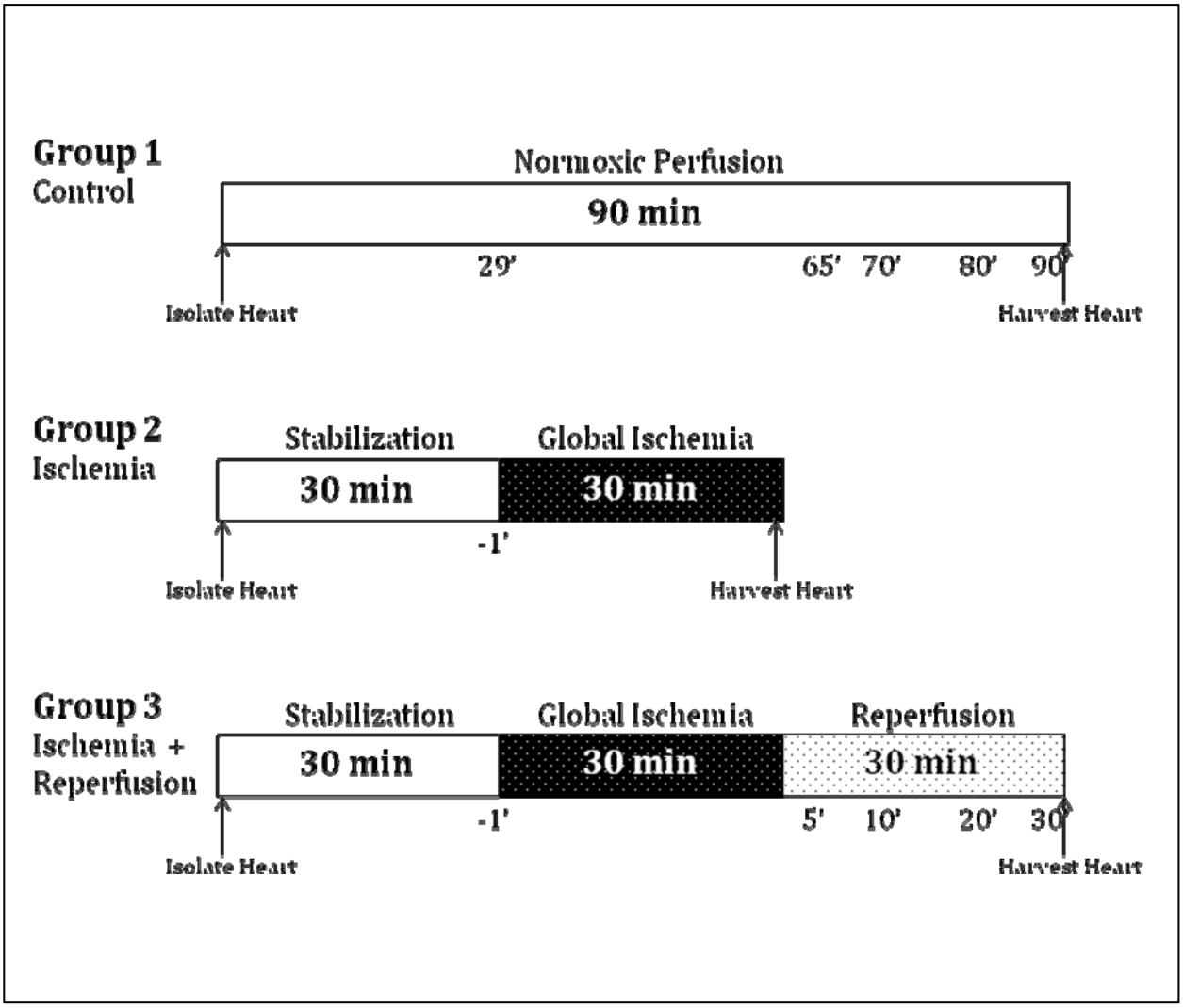


Figure 7: Langendorff isolated heart protocol for ischemia and I/R.
Experimental protocol for studies of PDE5 following ischemia and I/R. Arrows indicate the time points for performance of surgical procedures and minute marks show time points for collection of coronary effluent.

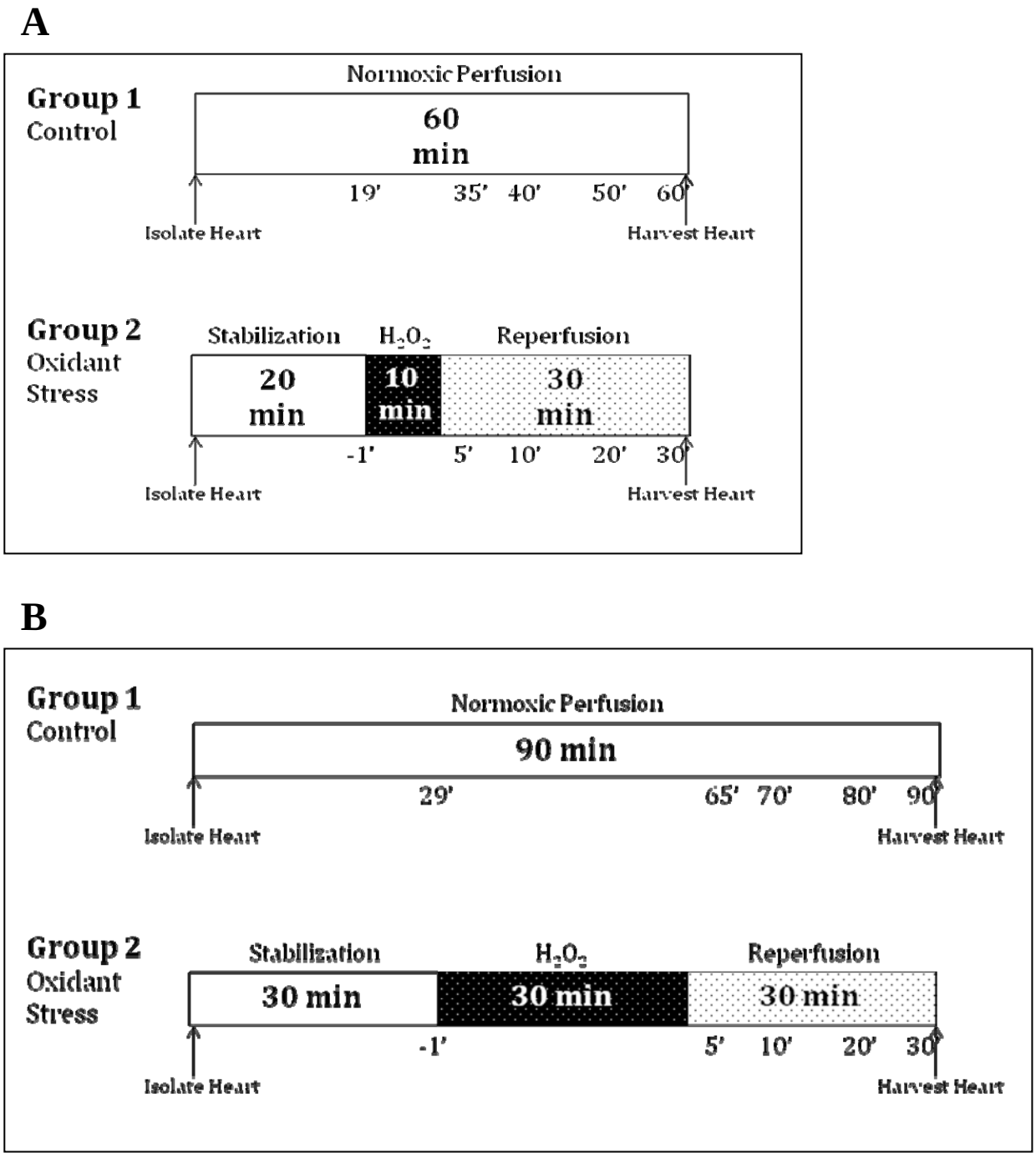


Figure 8: Langendorff isolated heart protocol for oxidant stress with 100 µM H_2O_2. Experimental protocol for studies of PDE5 following 10 minutes (A) or 30 minutes (B) of oxidant stress by perfusion with the oxygen radical generator H_2O_2. Arrows indicate the time points for performance of surgical procedures and minute marks show time points for collection of coronary effluent.

Results

3.1 Exclusions

Among the hearts used in the experimental protocols, approximately 25% 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, longer cannulation time (greater than 3 minutes), excessive coronary flow, and arrhythmic heart beat during the stabilization period.

3.2 Infarct size following Sildenafil preconditioning

The selective PDE5 inhibitor sildenafil has been shown to have a cardioprotective effect in the mouse heart model by results obtained from my lab group. Mice were divided into two experimental groups and sextuplicate samples were obtained following the surgical protocol for saline or sildenafil treatment, respectively (Fig. 4). Myocardial infarct size was significantly reduced from 41.8±1.5% in saline-treated control mice to 15.8±1.3% in sildenafil-treated mice (Fig. 9A, Fig. 10A & B). Infarct size was calculated as a percentage of total risk area, a measured area that did not vary between the two experimental groups (Fig. 9B).

3.3 In vivo ischemia and I/R

Mice were divided into three experimental groups and triplicate samples of the whole heart were harvested following 30 minutes of ischemia, 30 minutes of ischemia plus 12 hours reperfusion, or 30 minutes of ischemia plus 24 hours of reperfusion (Fig. 3).

Cardiac PDE5 expression: The expression of PDE5 was confirmed via Western blot analysis (Fig. 11). All three treatment groups elicited a significant upregulation of PDE5 protein when compared to their respective control sham operated groups (Fig. 12, Table 1). Interestingly, while this upregulation of PDE5 protein surged immediately following 30 minutes of ischemia, there was a decrease at 12 hours of reperfusion followed by a subsequent increase at 24 hours of reperfusion (Fig. 13).

Cardiac PDE5 enzyme activity: The cGMP hydrolytic activity was confirmed via spectrophotometric analysis. All three treatment groups showed a trend towards increase in the activity of PDE5 enzyme when compared to the respective control sham operated group (Fig. 14, Table 1).

Cardiac pPDE5 expression: The expression of pPDE5 was confirmed via western blot analysis (Fig. 15). After careful densitometry measurement of completed western blots, no appreciable difference in the phosphorylation state of PDE5 was observed between any of the time points (Fig. 16, Table 1).

Cardiac pVASP expression: The expression of pVASP was confirmed via western blot analysis (Fig. 17). Densitometric scanning showed no appreciable difference in the phosphorylation state of VASP versus total VASP between any of the time points (Fig. 18, Table 1).

3.4 Ex vivo ischemia and I/R

Mice were divided into three experimental groups and quadruplicate samples of the whole heart were harvested following 90 minutes of normoxic perfusion, 30 minutes of global ischemia, or 30 minutes of global ischemia plus 30 minutes of reperfusion (Fig. 7).

Cytotoxicity: The time course of LDH release into the coronary effluent during the pre-ischemic stabalization and reperfusion periods was measured by spectrophotometric analysis. Reperfusion of the isolated heart following ischemia caused a significant increase in the leakage of LDH (Fig. 19).

Cardiac PDE5 expression: The expression of PDE5 was confirmed via western blot analysis (Fig. 20). The treatment groups elicited a significant increase in the upregulation of PDE5 protein when compared to their respective control of 90 minutes normoxic perfusion (Fig. 21, Table 1).

Cardiac PDE5 enzyme activity: The cGMP hydrolytic activity was confirmed via spectrophotometric analysis. Both treatment groups trended to increase in the activity of PDE5 enzyme when compared to the respective control normoxic perfusion group (Fig. 22, Table 1).

Cardiac pPDE5 expression: The expression of pPDE5 was confirmed via western blot analysis (Fig. 23). No appreciable difference in the phosphorylation state of PDE5 was observed between any of the time points (Fig. 24, Table 1).

Cardiac pVASP expression: The expression of pVASP was confirmed via western blot analysis (Fig. 25). Interestingly, treatment of 30 minutes of global ischemia elicited a

significant increase in the upregulation of pVASP protein when compared to the respective normoxic perfusion, however no appreciable difference in the phosphorylation state of VASP versus total VASP was observed when the 30 minutes of global ischemia was followed by 30 minutes of reperfusion (Fig. 26, Table 1).

3.5 10 minutes of ex vivo oxidant stress

Mice were divided into two experimental groups and sextuplicate samples of whole mouse heart were harvested following 60 minutes of normoxic perfusion or 10 minutes of perfusion with H_2O_2 plus 30 minutes of reperfusion (Fig. 8a).

Cytotoxicity: The time course of LDH release into the coronary effluent during the stabalization and reperfusion periods was measured by spectrophotometric analysis. Perfusion of the isolated heart with H_2O_2 did not cause a significant increase in the leakage of LDH (Fig. 27).

Cardiac PDE5 expression: The expression of PDE5 was confirmed via western blot analysis (Fig. 28). After careful densitometry measurement of completed western blots, no appreciable difference in the expression of PDE5 was observed (Fig. 29, Table 1).

Cardiac PDE5 enzyme activity: The cGMP hydrolytic activity was confirmed via spectrophotometric analysis. Oxidant stress had little effect on the activity of PDE5 enzyme when compared to the respective control normoxic perfusion group (Fig. 30, Table 1).

Cardiac pPDE5 expression: The expression of pPDE5 was confirmed via western blot analysis (Fig. 31). No appreciable difference in the phosphorylation state of PDE5 was observed between groups (Fig. 32, Table 1).

Cardiac pVASP expression: The expression of pVASP was confirmed via western blot analysis (Fig. 33). After careful densitometry measurement of completed western blots, no appreciable difference in the expression of pVASP was observed (Fig. 34, Table 1).

3.6 30 minutes of ex vivo oxidant stress

Mice were divided into two experimental groups and duplicate samples of whole hearts were harvested following 90 minutes of normoxic perfusion or 30 minutes of perfusion with H_2O_2 plus 30 minutes of reperfusion (Fig. 8b).

Cytotoxicity: The time course of LDH release into the coronary effluent of isolated mouse hearts during the stabalization and reperfusion periods was measured by spectrophotometric analysis. Perfusion of the isolated heart with H_2O_2 caused a significant increase in the leakage of LDH (Fig. 35).

Cardiac PDE5 expression: The expression of PDE5 was confirmed via western blot analysis (Fig. 36). Densitometric analysis of blots showed no appreciable difference in the expression of PDE5 (Fig. 37, Table 1).

Cardiac PDE5 enzyme activity: The cGMP hydrolytic activity was confirmed via spectrophotometric analysis. Oxidant stress had little effect on the activity of PDE5 enzyme when compared to the respective control normoxic perfusion group (Fig. 38, Table 1).

Cardiac pPDE5 expression: The expression of pPDE5 was confirmed via western blot analysis (Fig. 39). No appreciable difference in the phosphorylation state of PDE5 was observed between groups (Fig. 40, Table 1).

Cardiac pVASP expression: The expression of pVASP was confirmed via western blot analysis (Fig. 41). 30 minutes of oxidant stress by H_2O_2 perfusion elicited a significant upregulation of pVASP protein expression when compared to the respective normoxic perfusion (Fig. 42, Table 1).

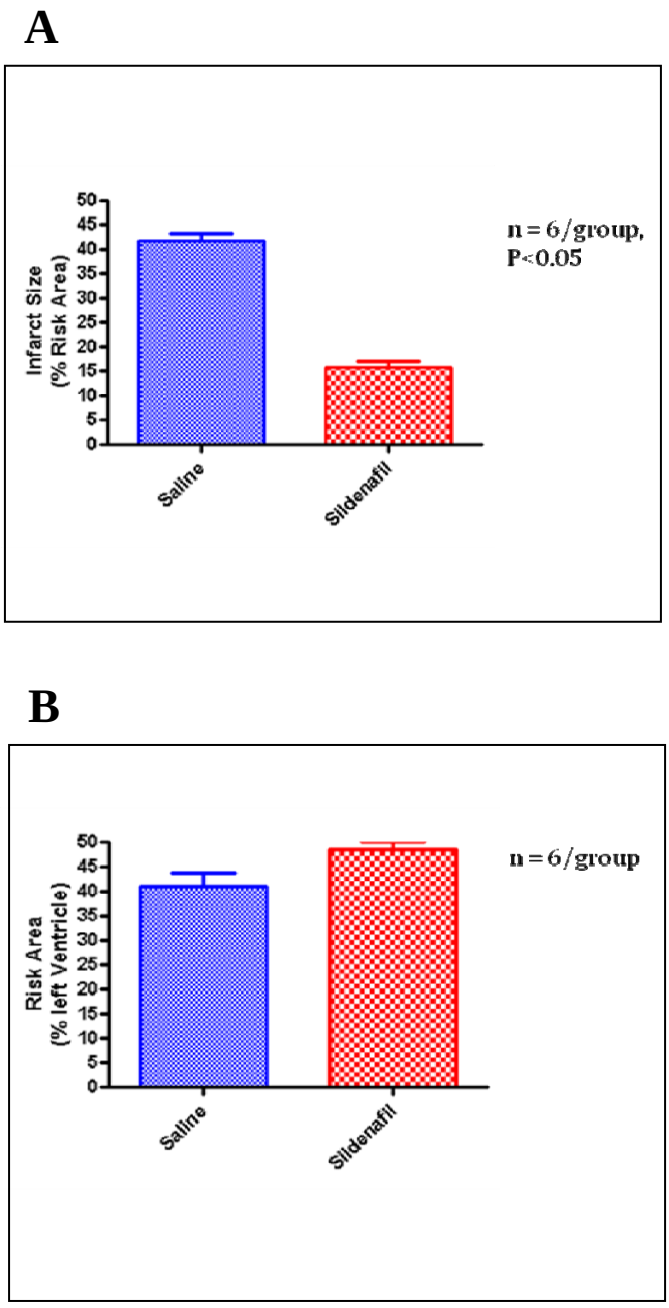
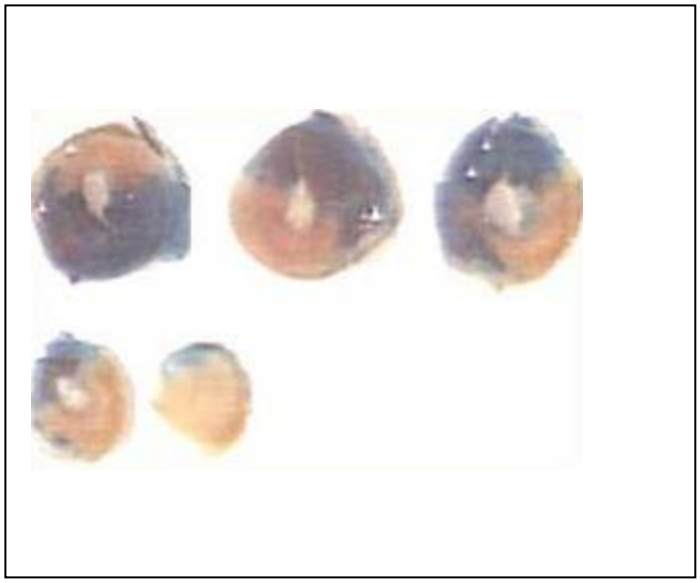


Figure 9: Sildenafil preconditioning reduces infarct size
Adult male ICR mice were treated with either saline or sildenafil intraperitoneally 1 hour before being subjected to 30 minutes of left anterior descending coronary artery ligation followed by 24 hours of reperfusion. Infarct size was significantly reduced by sildenafil (A), while risk area was calculated as a percentage of total area and was similar in both groups (B).

A



B

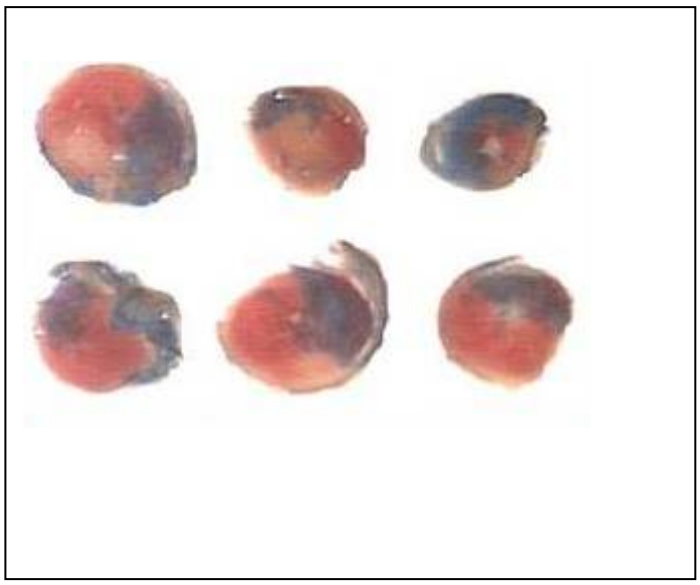


Figure 10: Representative digital images of stained heart slices
Heart slices were stained with Evans blue dye, TTC, and fixed with formalin. Unaffected, non-risk area shows dark blue, viable risk area tissue remains red, and infarcted tissue appears pale white. The saline control heart (A) has a much larger infarcted area when compared to the sildenafil-treated heart (B), which shows healthy myocardium and little infarcted tissue.

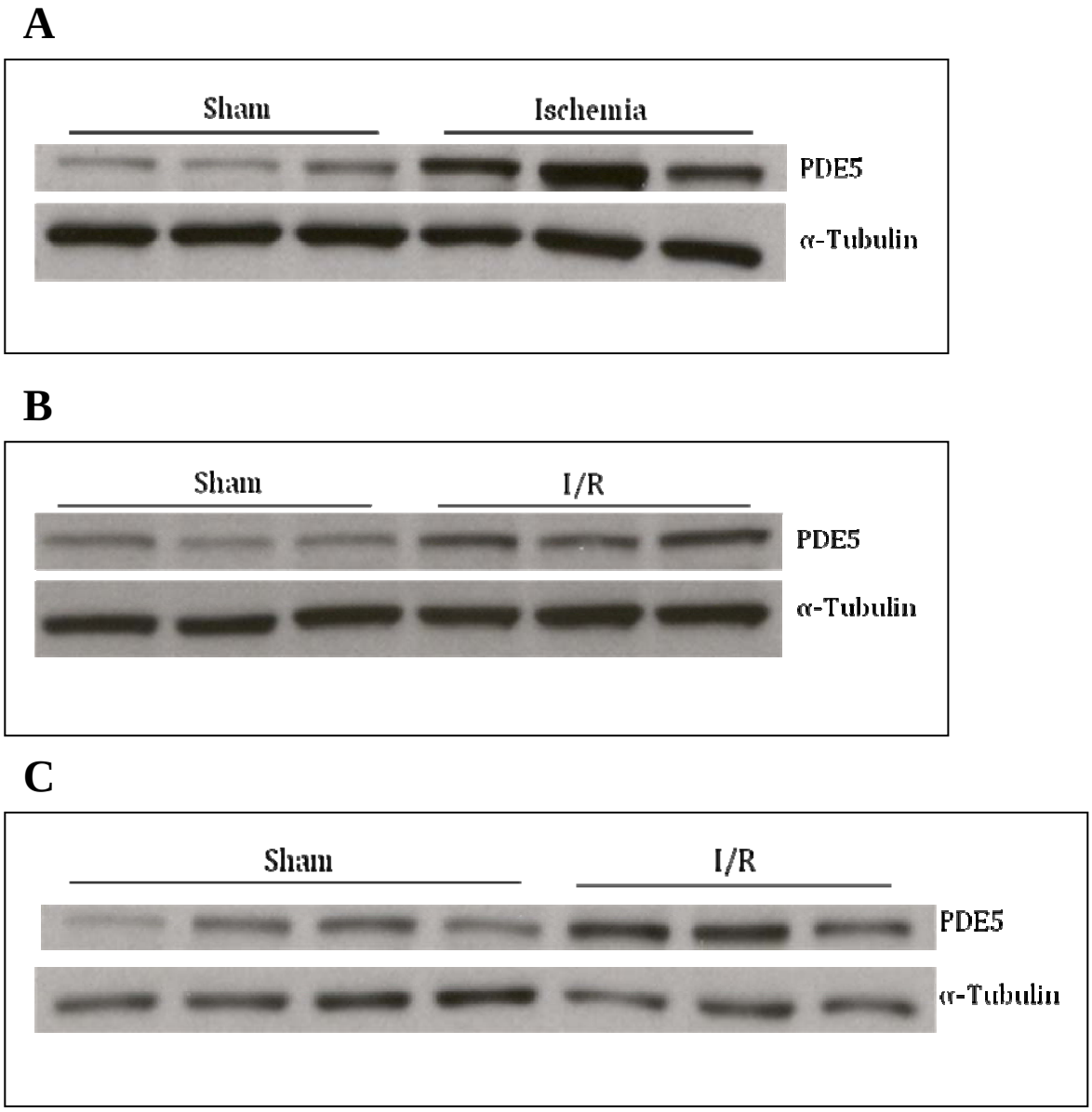


Figure 11: Ischemia and I/R upregulate cardiac expression of PDE5 *in vivo*
Adult male ICR mice were subjected to 30 minutes of left anterior descending coronary artery ligation alone (A), or followed by 12 hours of reperfusion (B), or 24 hours of reperfusion (C). Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by Western blotting with antibody against PDE5 (98 kDa). Blots were reprobed for α-Tubulin (55 kDa) to demonstrate equal protein loading.

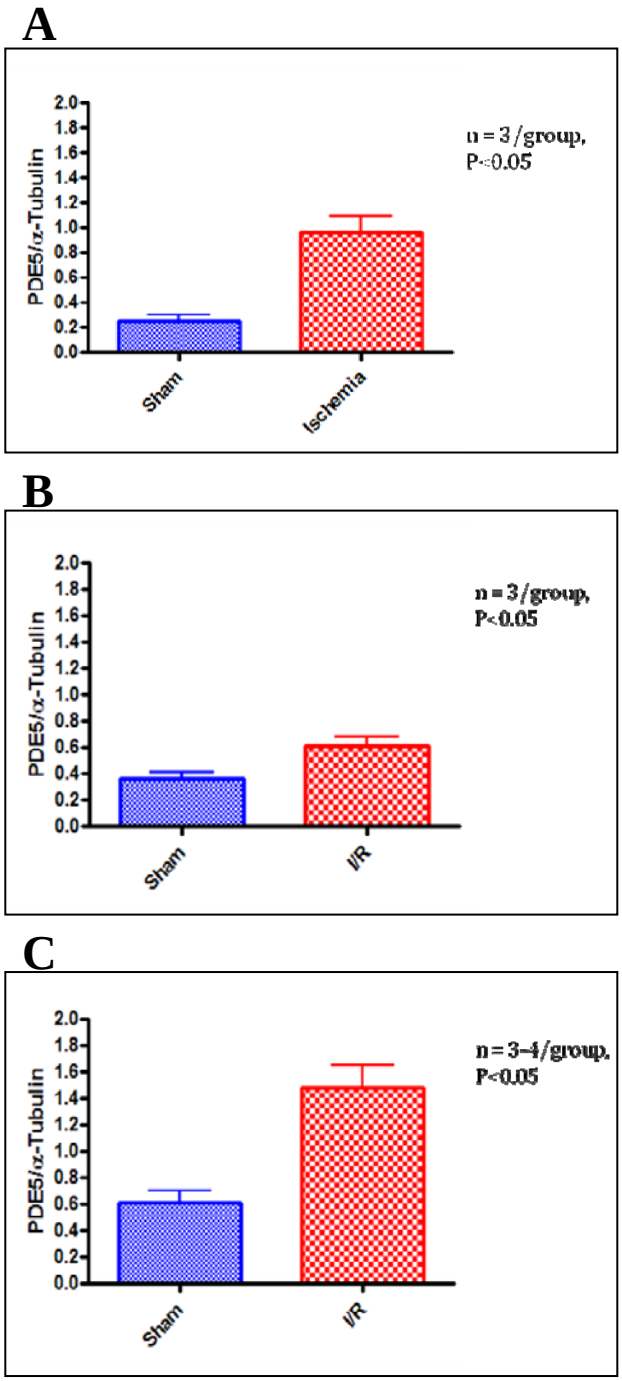


Figure 12: Ischemia and I/R significantly increase cardiac PDE5 protein expression *in vivo*.
Adult male ICR mice were subjected to 30 minutes of left anterior descending coronary artery ligation alone(A), or followed by 12 hours of reperfusion (B), or 24 hours of reperfusion (C). Western blot densitometry measurements were calculated as a ratio of PDE5 to α-Tubulin which is significantly increased by both ischemia and I/R when compared to respective sham controls. Bar graphs represent the mean ± SEM.

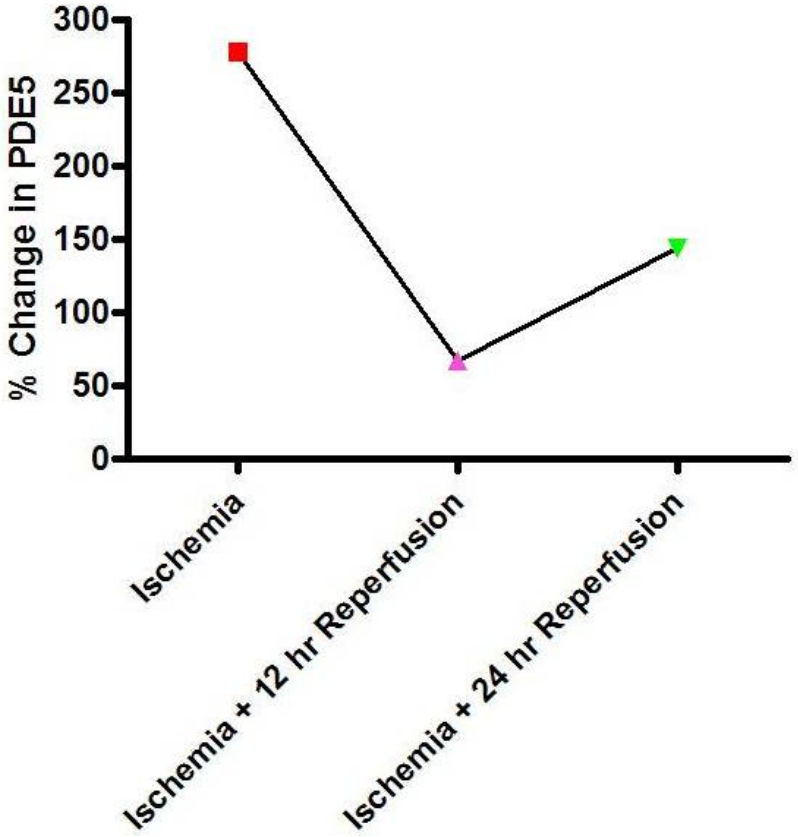


Figure 13: PDE5 protein expression varies with duration of reperfusion *in vivo*. In terms of the % increase from respective sham controls, the upregulation of PDE5 protein peaked immediately following 30 minutes of ischemia, with a decrease at 12 hours of reperfusion followed by a subsequent increase at 24 hours of reperfusion.

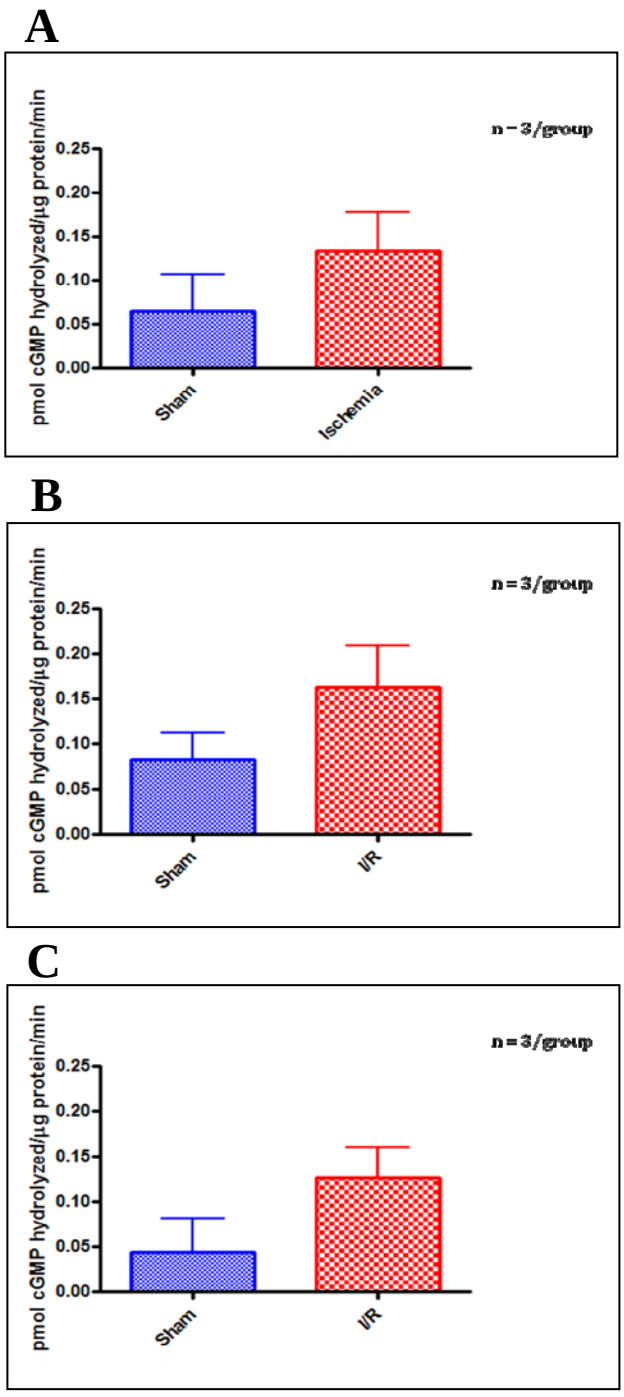


Figure 14: Ischemia and I/R trend to increase cardiac PDE5 protein activity *in vivo*. Adult male ICR mice were subjected to 30 minutes of left anterior descending coronary artery ligation alone (A), or followed by 12 hours of reperfusion (B), or 24 hours of reperfusion (C). Hearts were harvested for total protein which was assayed for cGMP hydrolytic activity. PDE5-specific activity was measured as the sildenafil-inhibitable fraction of total cGMP hydrolysis, normalized for total micrograms of protein, and trends to increase with both ischemia and I/R when compared to the respective sham controls. Results are shown as means ± SEM (n=3-4; read in duplicate).

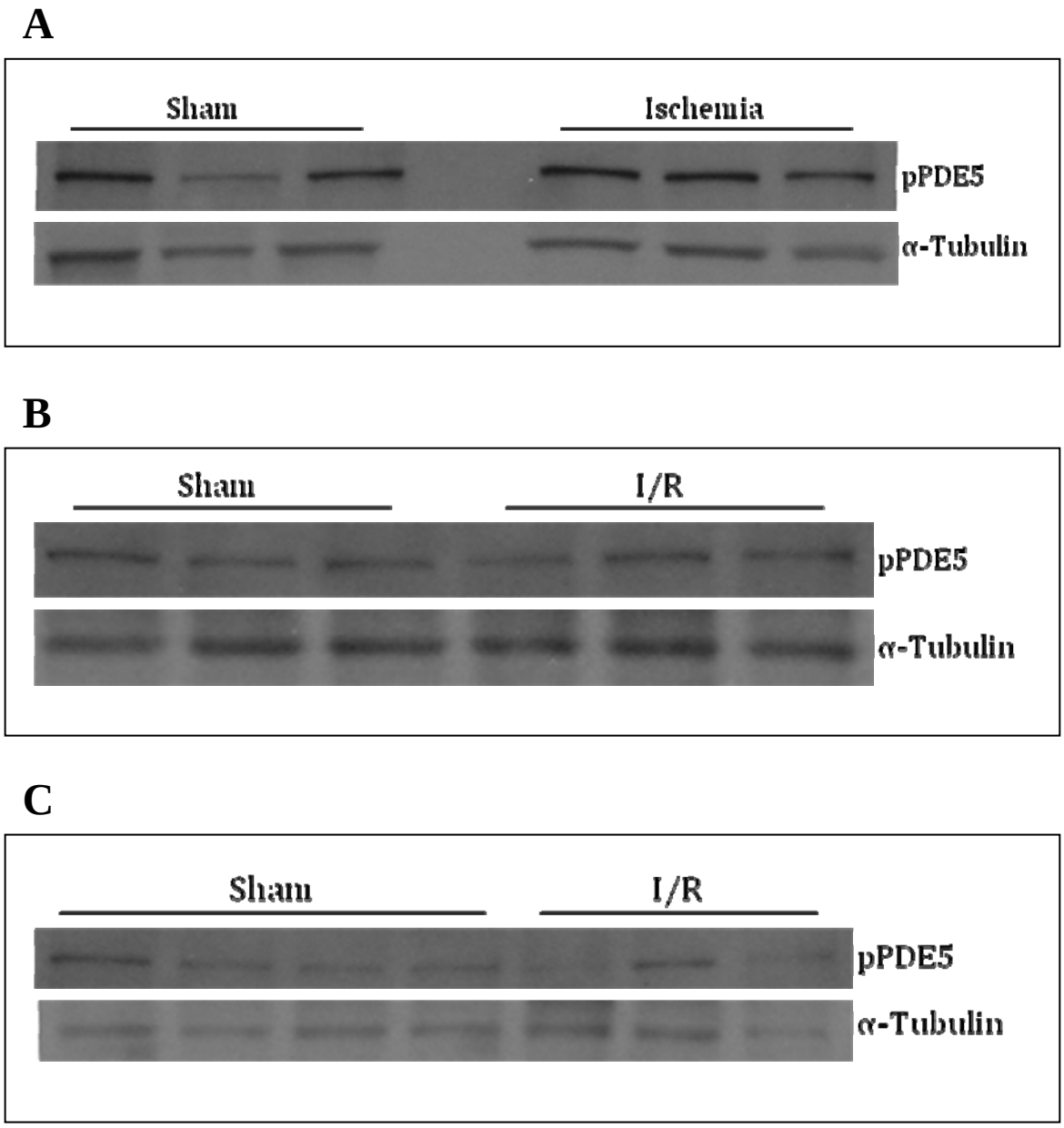


Figure 15: Ischemia and I/R do not affect cardiac pPDE5 expression *in vivo*. Adult male ICR mice were subjected to 30 minutes of left anterior descending coronary artery ligation alone (A), or followed by 12 hours of reperfusion (B), or 24 hours of reperfusion (C). Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by western blotting with antibody against pPDE5 (120 kDa). Blots were reprobed for α-Tubulin (55 kDa) to demonstrate equal protein loading.

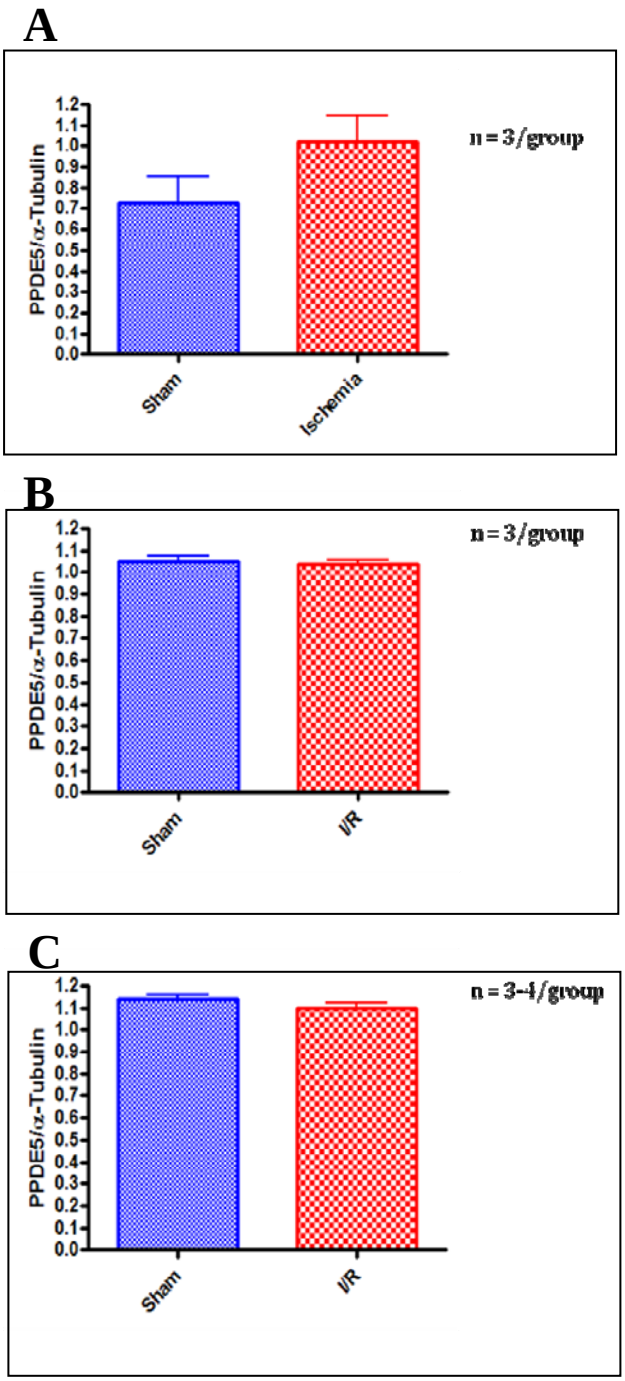


Figure 16: Ischemia and I/R do not significantly affect cardiac pPDE5 protein expression *in vivo*.
Adult male ICR mice were subjected to 30 minutes of left anterior descending coronary artery ligation alone (A), or followed by 12 hours of reperfusion (B), or 24 hours of reperfusion (C). Western blot densitometry measurements were calculated as a ratio of pPDE5 to α-Tubulin which is not significantly changed by either ischemia or I/R when compared to respective sham controls. Bar graphs represent the mean ± SEM.

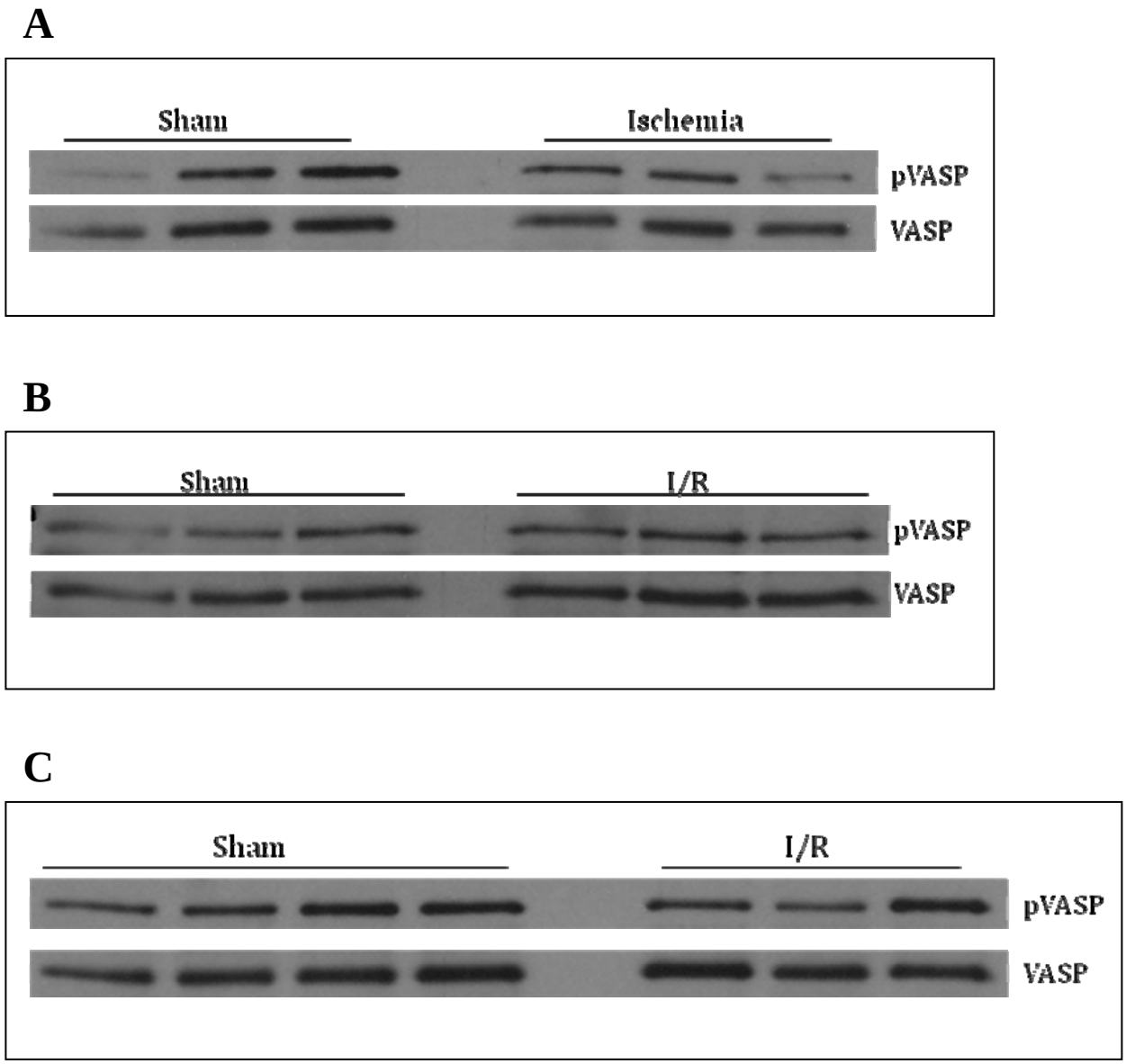


Figure 17: Ischemia and I/R do not affect cardiac pVASP expression *in vivo*. Adult male ICR mice were subjected to 30 minutes of left anterior descending coronary artery ligation alone (A), or followed by 12 hours of reperfusion (B), or 24 hours of reperfusion (C). Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by western blotting with antibody against pVASP (48 kDa). Blots were reprobed for total VASP (46 kDa) to ensure that any changes in phosphorylation of VASP were not due to changes in its total protein levels.

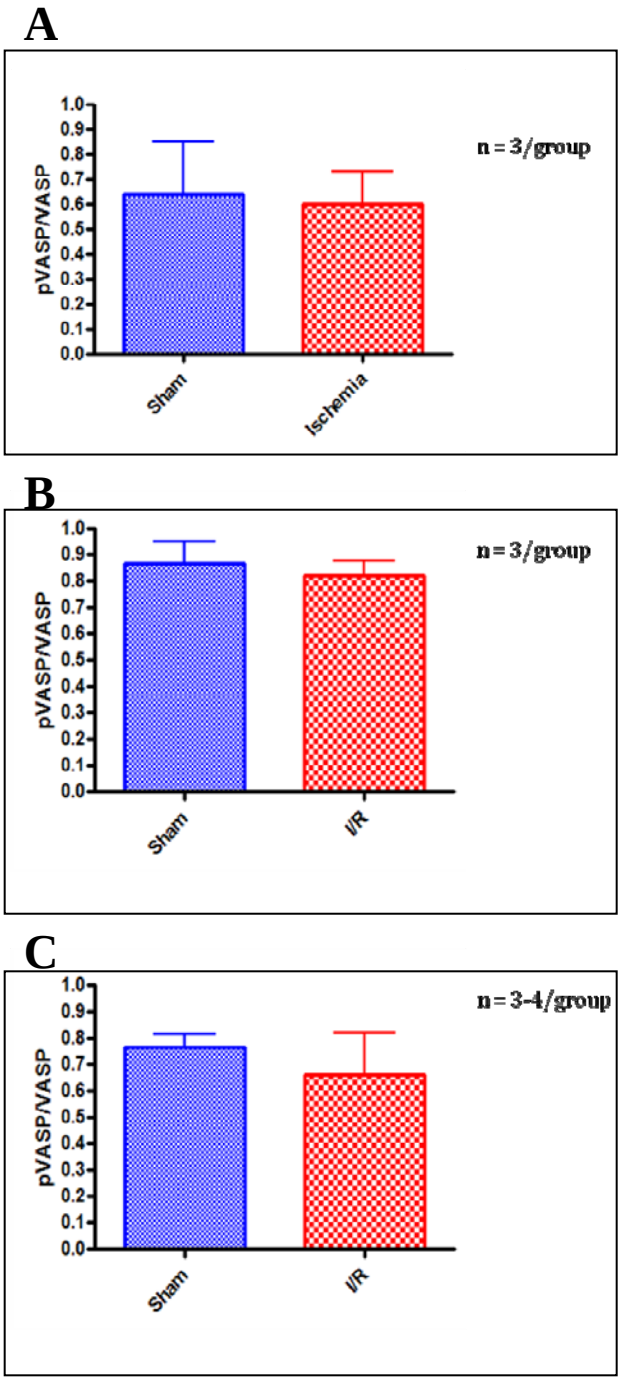


Figure 18: Ischemia and I/R do not significantly affect cardiac pVASP protein expression *in vivo*.
Adult male ICR mice were subjected to 30 minutes of left anterior descending coronary artery ligation alone (A), or followed by 12 hours of reperfusion (B), or 24 hours of reperfusion (C). Western blot densitometry measurements were calculated as a ratio of pVASP to total VASP which is not significantly changed by either ischemia or I/R when compared to respective sham controls. Bar graphs represent the mean ± SEM.

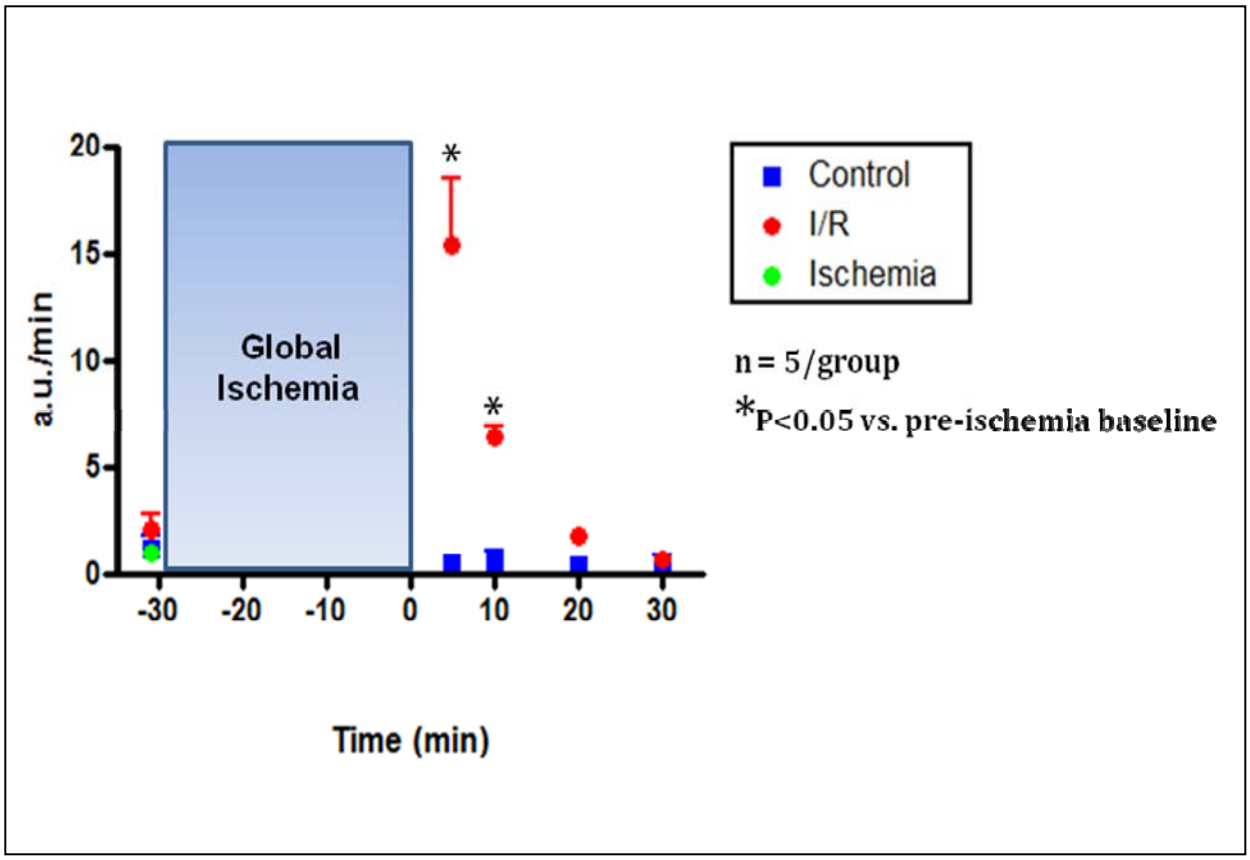


Figure 19: Leakage of intracellular LDH due to I/R.
Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion, 30 minutes of global ischemia, or 30 minutes of global ischemia plus 30 minutes of reperfusion. Leakage of LDH in heart effluent perfusate was measured before and after the 30 minutes of global ischemia (except in the case of ischemia treatment alone, which by definition lacks reperfusion measurement). Measurements of LDH activity were done spectrophotometrically using a commercially available enzyme assay kit at 490 nm wavelength (expressed as arbitrary unit, a.u./mL) and normalized against the individual coronary flow rate (mL/min). Reperfusion of the isolated heart following ischemia caused a significant increase in the leakage of LDH. Results are shown as means ± SEM.

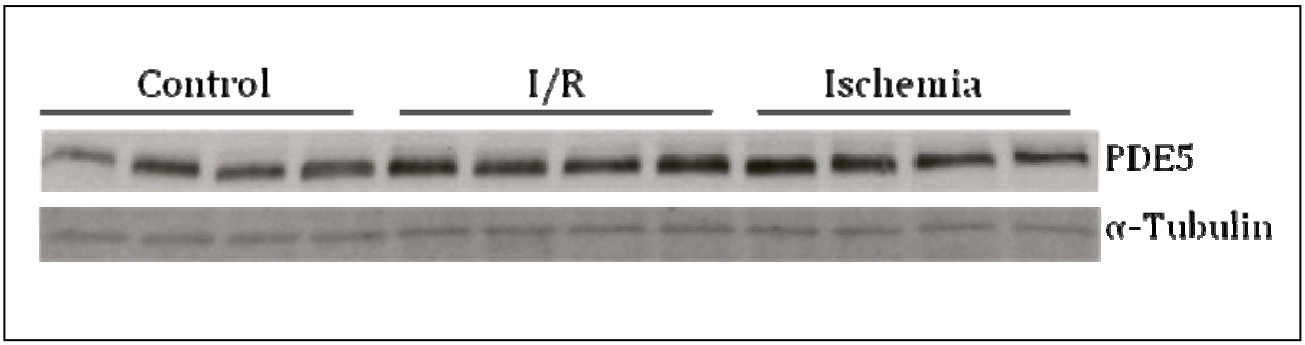


Figure 20: Ischemia and I/R upregulate cardiac expression of PDE5 *ex vivo*.
Adult male ICR mouse hearts were isolated and subjected to 90 minutes of normoxic perfusion, or 30 minutes of global zero-flow ischemia, or 30 minutes of global zero-flow ischemia plus 30 minutes of reperfusion in Langendorff mode. Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by Western blotting with antibody against PDE5 (98 kDa). Blots were reprobed for α-Tubulin (55 kDa) to demonstrate equal protein loading.

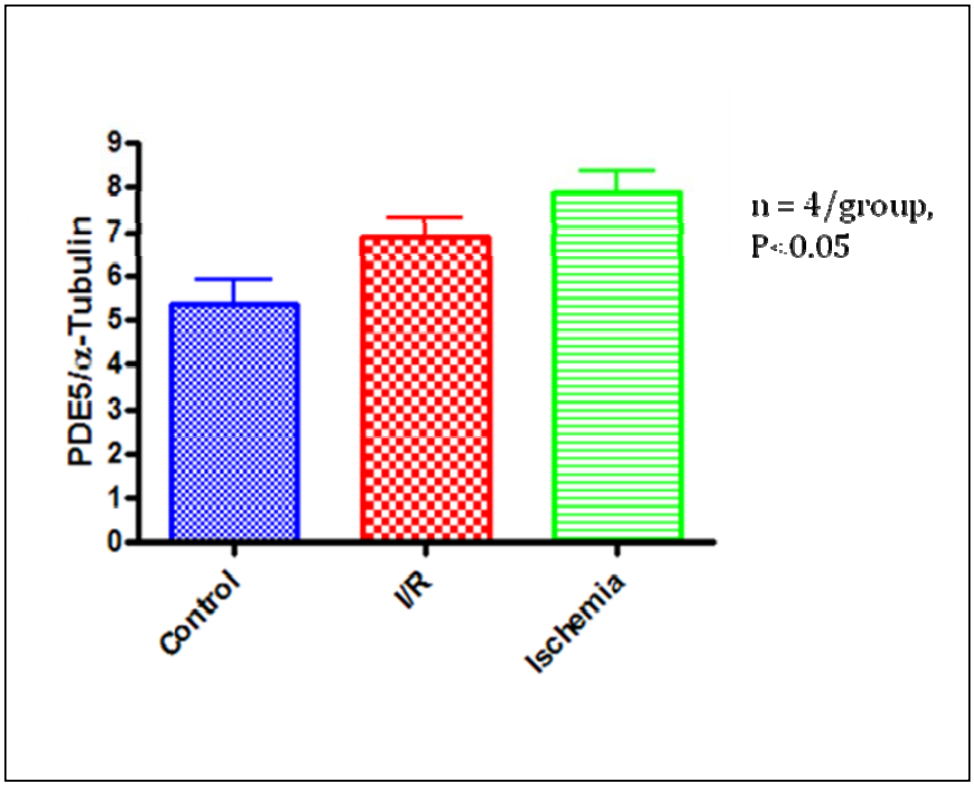


Figure 21: Ischemia and I/R significantly increase cardiac PDE5 protein expression *ex vivo*.
Adult male ICR mouse hearts were isolated and subjected to 90 minutes of normoxic perfusion, 30 minutes of global ischemia, or 30 minutes of global ischemia plus 30 minutes of reperfusion. Western blot densitometry measurements were calculated as a ratio of PDE5 to α-Tubulin which is significantly increased by both ischemia and ischemia/reperfusion when compared to respective normoxic perfusion controls. Bar graphs represent the mean ± SEM.

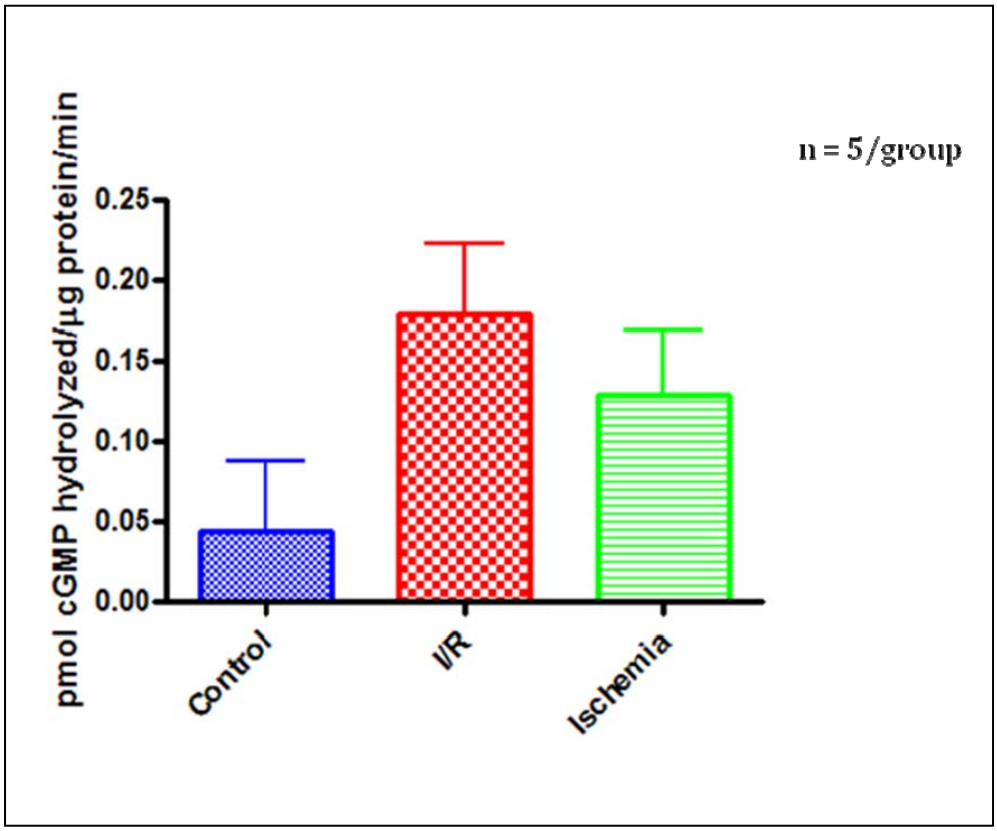


Figure 22: Ischemia and I/R trend to increase cardiac PDE5 protein activity *ex vivo*. Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion, 30 minutes of global ischemia, or 30 minutes of global ischemia plus 30 minutes of reperfusion. Hearts were harvested for total protein which was assayed for cGMP hydrolytic activity. PDE5-specific activity was measured as the sildenafil-inhibitable fraction of total cGMP hydrolysis, normalized for total micrograms of protein, and trends to increase with both ischemia and reperfusion when compared to the respective control normoxic perfusion group. Results are shown as means ± SEM (n=5; read in duplicate).

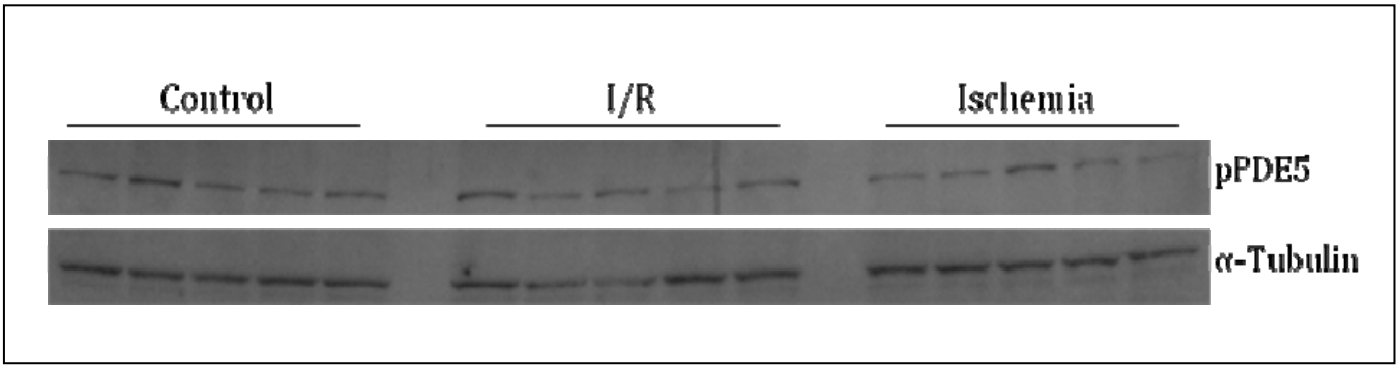


Figure 23: Ischemia and I/R do not affect cardiac pPDE5 expression *ex vivo*. Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion, 30 minutes of global ischemia, or 30 minutes of global ischemia plus 30 minutes of reperfusion. Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by western blotting with antibody against pPDE5 (120 kDa). Blots were reprobed for α-Tubulin (55 kDa) to demonstrate equal protein loading.

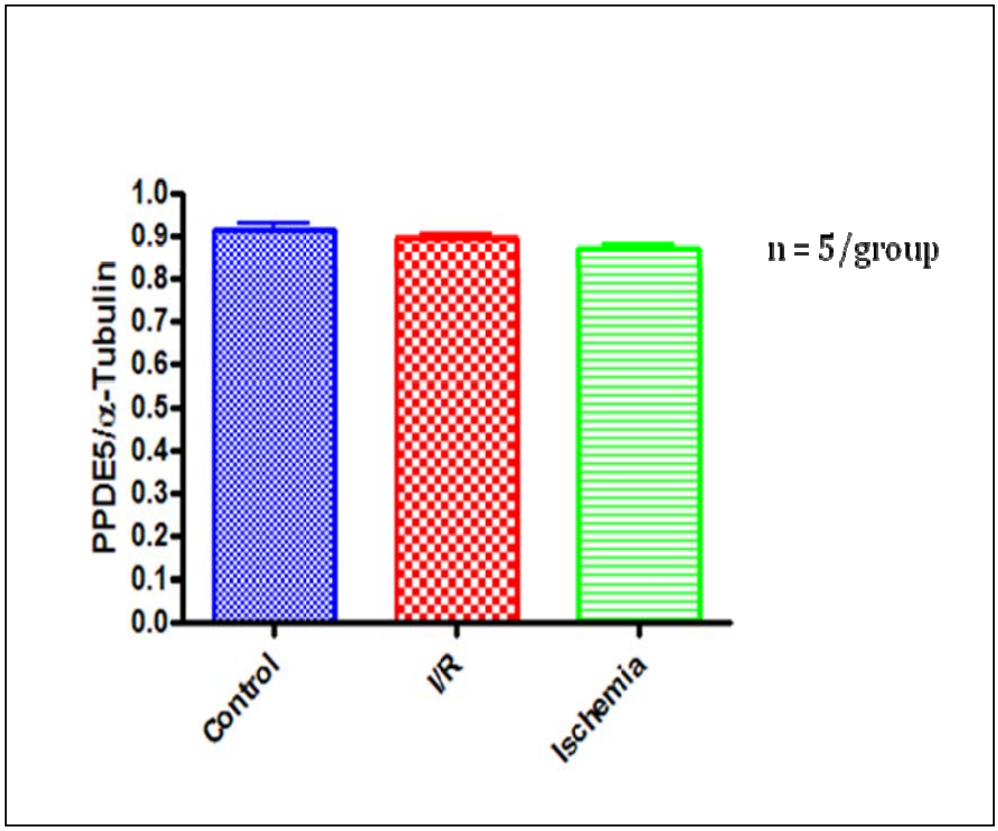


Figure 24: Ischemia and I/R do not significantly affect cardiac pPDE5 protein expression *ex vivo.*
Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion, 30 minutes of global ischemia, or 30 minutes of global ischemia plus 30 minutes of reperfusion. Western blot densitometry measurements were calculated as a ratio of pPDE5 to α-Tubulin which is not significantly changed by either ischemia or ischemia/reperfusion when compared to respective normoxic perfusion. Bar graphs represent the mean ± SEM.

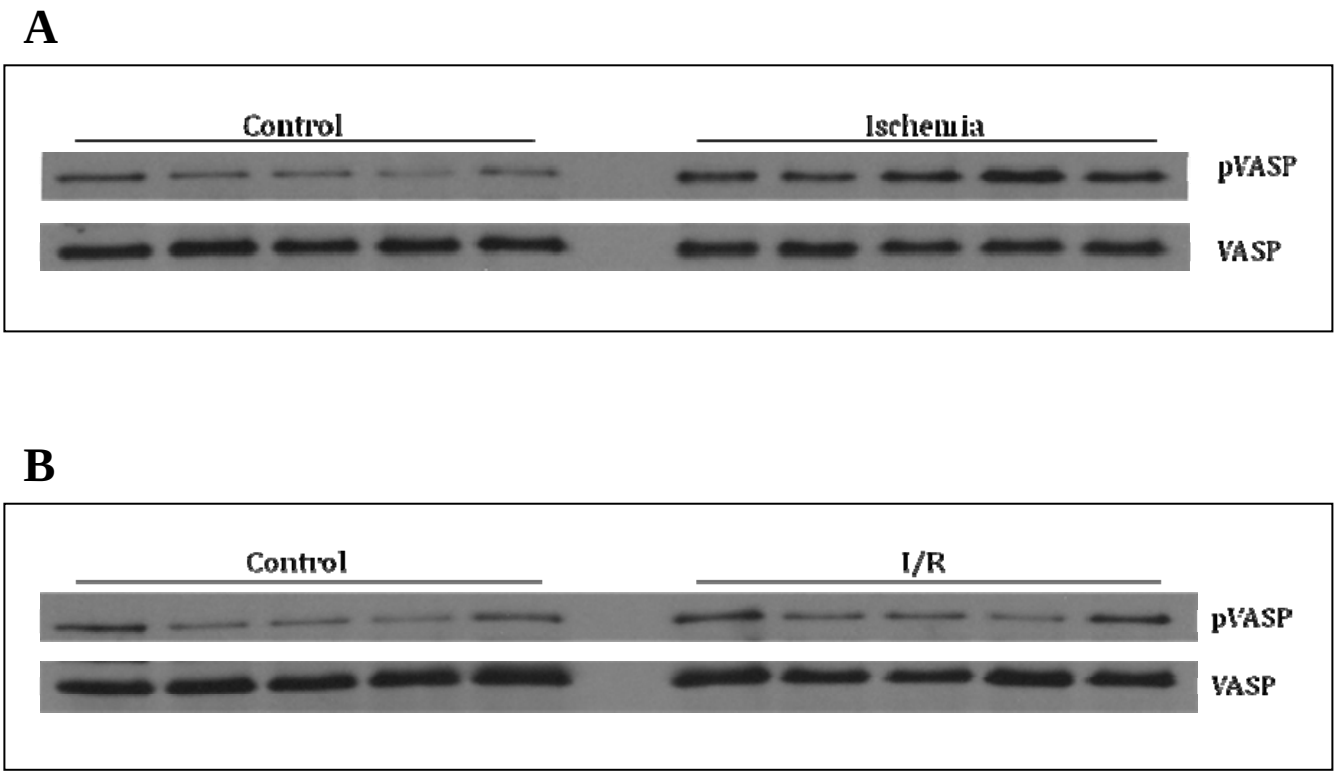


Figure 25: Ischemia upregulates expression of cardic pVASP *ex vivo* which is partially normalized by reperfusion.
Adult male ICR mouse hearts were isolated and subjected to 90 minutes of normoxic perfusion as a control, 30 minutes of global ischemia (A), or 30 minutes of global ischemia plus 30 minutes of reperfusion (B). Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by western blotting with antibody against pVASP (48 kDa). Blots were reprobed for total VASP (46 kDa) to ensure that any changes in phosphorylation of VASP were not due to changes in its total protein levels.

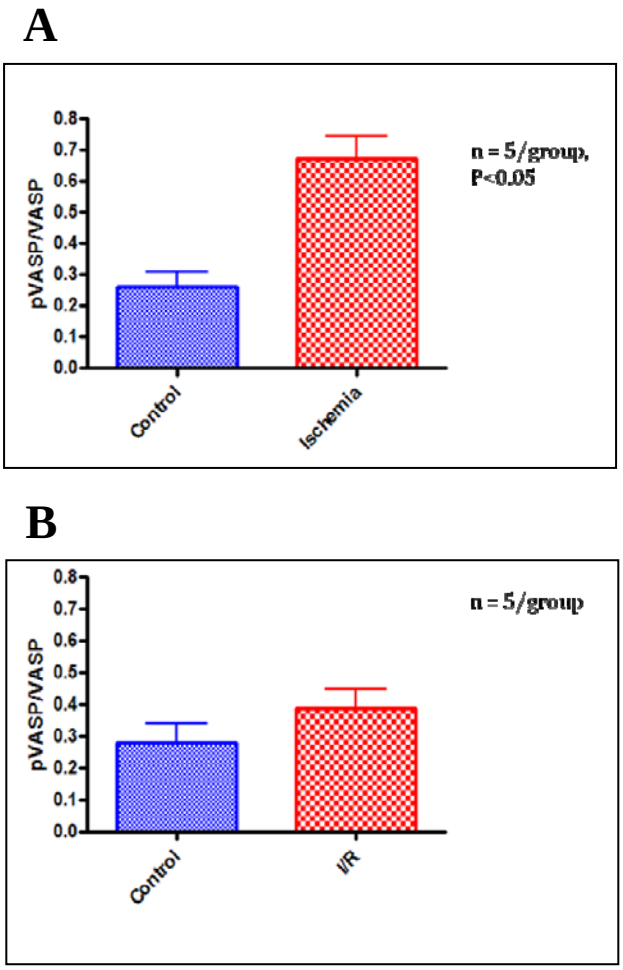


Figure 26: Ischemia significantly increases cardiac pVASP protein expression *ex vivo* which is partially normalized by reperfusion.
Adult male ICR mouse hearts were isolated and subjected to 90 minutes of normoxic perfusion as a control, 30 minutes of global ischemia (A), or 30 minutes of global ischemia plus 30 minutes of reperfusion (B). Western blot densitometry measurements were calculated as a ratio of pVASP to total VASP which is significantly increased following 30 minutes of ischemia when compared to respective normoxic perfusion (see Graph A). This increase is partially abolished by 30 minutes of reperfusion (see Graph B). Bar graphs represent the mean ± SEM.

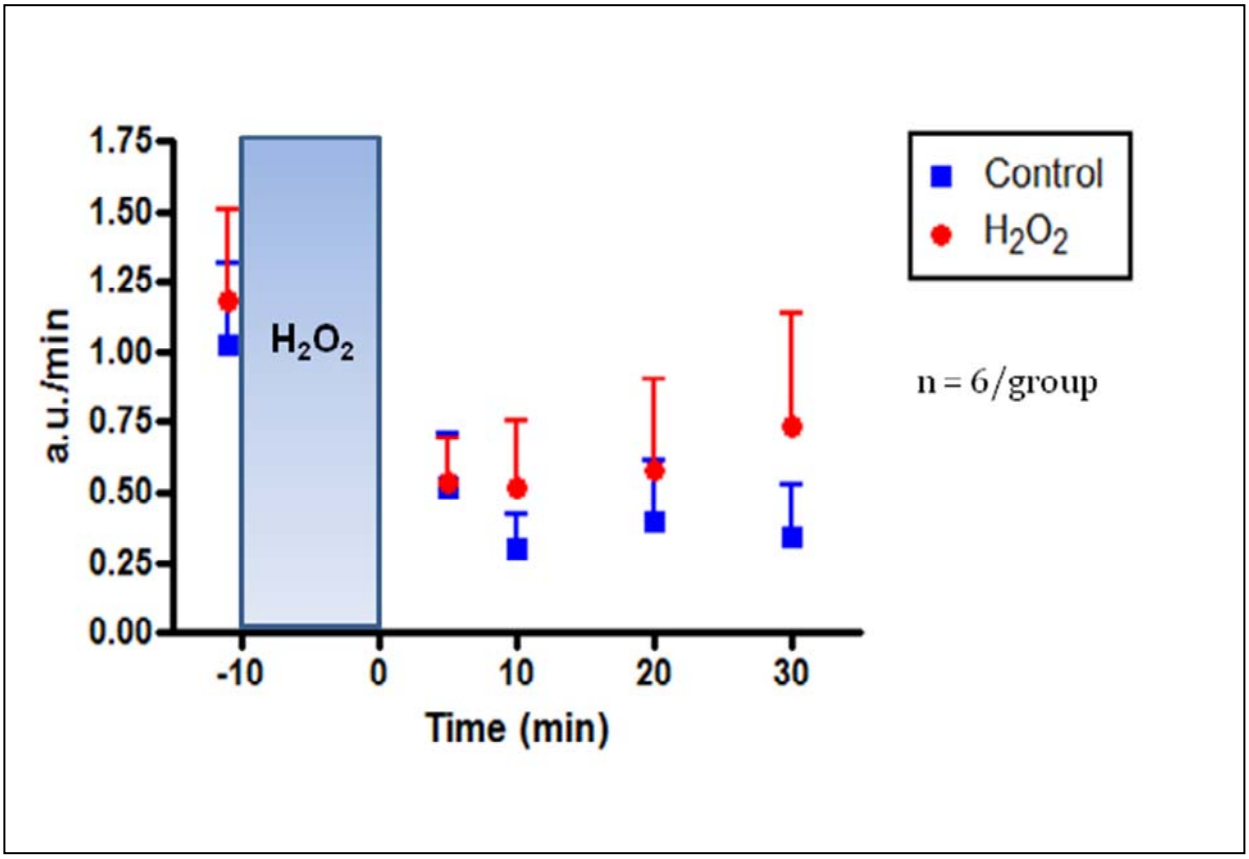


Figure 27: Leakage of intracellular LDH due to 10 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 infusion.
Adult male ICR mice hearts were isolated and subjected to 60 minutes of normoxic perfusion, or 10 minutes perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Leakage of LDH in heart effluent perfusate was measured before and after the 10 minutes of perfusion with H_2O_2. Measurements of LDH activity were done spectrophotometrically using a commercially available enzyme assay kit at 490 nm wavelength (expressed as arbitrary unit, a.u./mL) and normalized against the individual coronary flow rate (mL/min). 10 min of H_2O_2 infusion did not cause a significant increase in the leakage of LDH. Results are shown as means ± SEM.

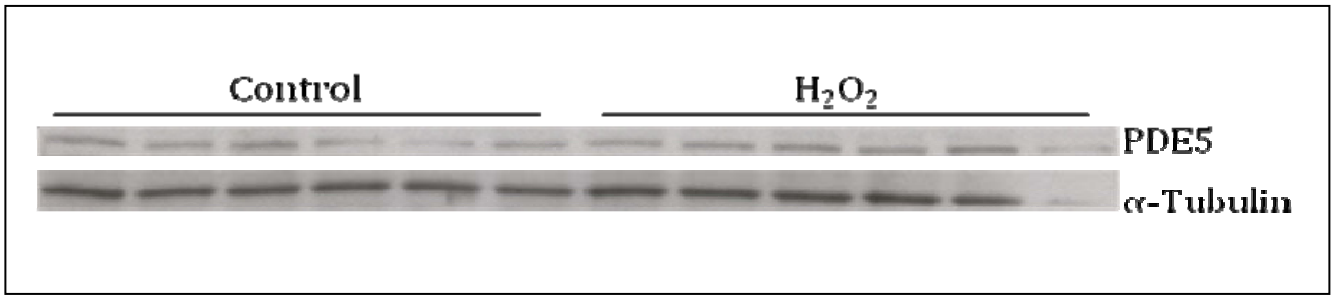


Figure 28: 10 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 does not affect cardiac expression of PDE5.
Adult male ICR mice hearts were isolated and subjected to 60 minutes of normoxic perfusion or 10 minutes of perfusion with the oxygen radical generator H_2O_2 (100 µM) followed by 30 minutes of washout normoxic perfusion. Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by western blotting with antibody against PDE5 (98 kDa). Blots were reprobed for α-Tubulin (55 kDa) to demonstrate equal protein loading.

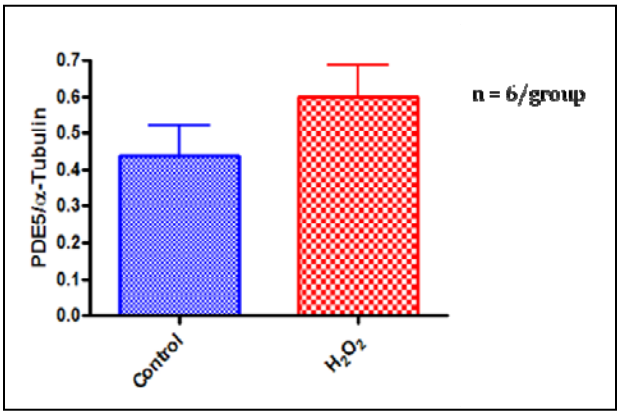


Figure 29: 10 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 does not significantly affect cardiac PDE5 protein expression.
Adult male ICR mice hearts were isolated and subjected to 60 minutes of normoxic perfusion or 10 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Western blot densitometry measurements were calculated as a ratio of PDE5 to α-Tubulin which is not significantly changed following oxidant stress when compared to respective normoxic perfusion control. Bar graphs represent the mean ± SEM.

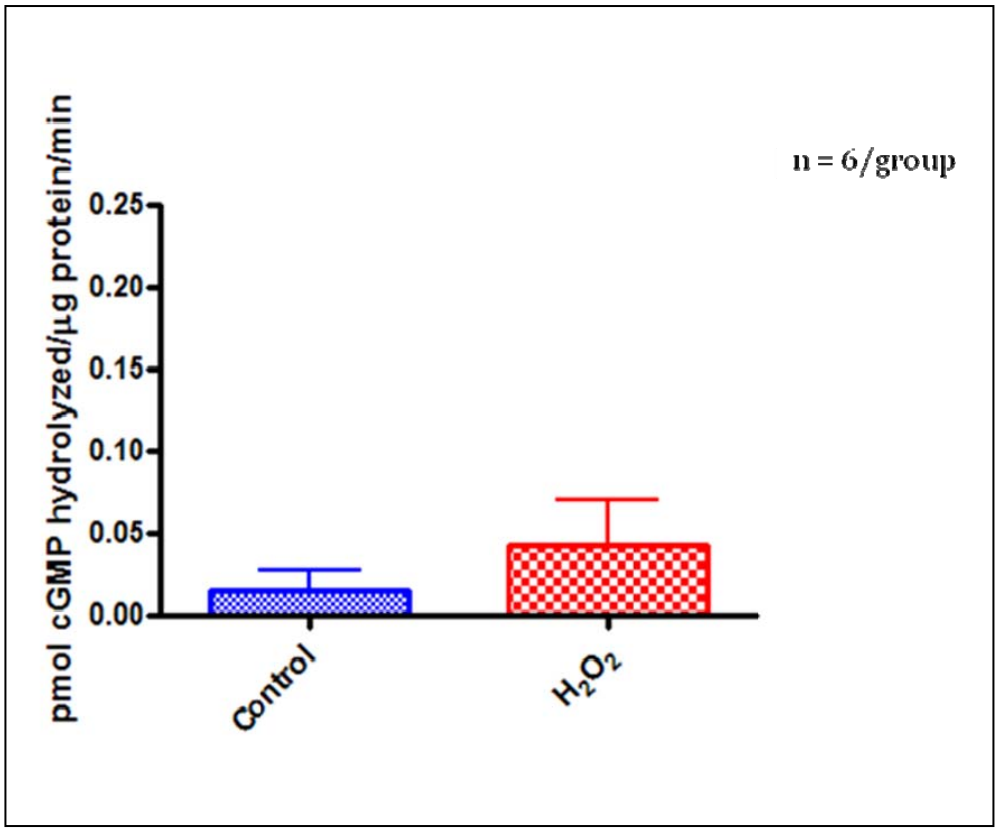


Figure 30: 10 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 does not affect cardiac PDE5 activity.
Adult male ICR mice hearts were isolated and subjected to 60 minutes of normoxic perfusion or 10 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Hearts were harvested for total protein which was assayed for cGMP hydrolytic activity. PDE5-specific activity was measured as the sildenafil-inhibitable fraction of total cGMP hydrolysis, normalized for total micrograms of protein, and is not changed when compared to the respective control normoxic perfusion group. Results are shown as means ± SEM (n=6; read in duplicate).

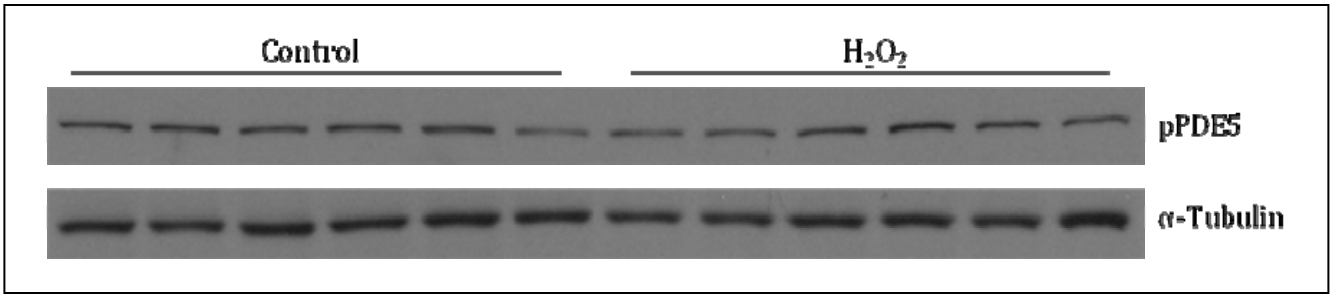


Figure 31: 10 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 does not affect cardiac expression of pPDE5.
Adult male ICR mice hearts were isolated and subjected to 60 minutes of normoxic perfusion or 10 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by western blotting with antibody against pPDE5 (120 kDa). Blots were reprobed for α-Tubulin (55 kDa) to demonstrate equal protein loading.

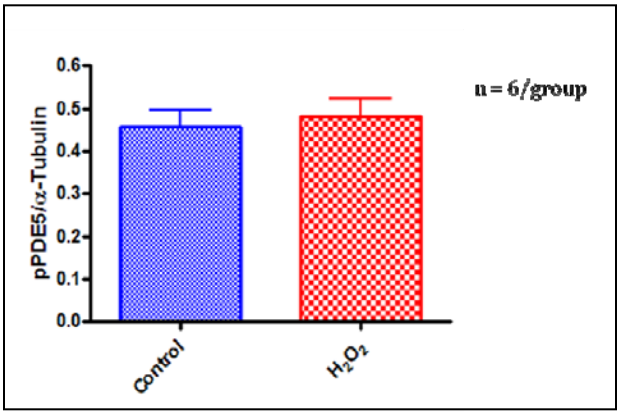


Figure 32: 10 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 does not significantly affect cardiac pPDE5 protein expression.
Adult male ICR mice hearts were isolated and subjected to 60 minutes of normoxic perfusion or 10 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Western blot densitometry measurements were calculated as a ratio of pPDE5 to α-Tubulin which is not changed following oxidant stress when compared to respective normoxic perfusion controls. Bar graphs represent the mean ± SEM.

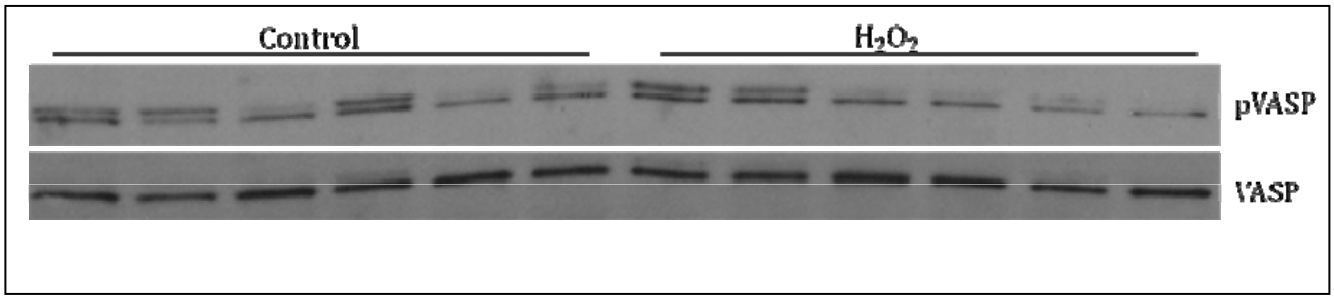


Figure 33: 10 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 does not affect cardiac expression of pVASP.
Adult male ICR mice hearts were isolated and subjected to 60 minutes of normoxic perfusion or 10 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by western blotting with antibody against pVASP (48 kDa, upper band). Blots were reprobed for total VASP (46 kDa) to ensure that any changes in phosphorylation of VASP were not due to changes in its total protein levels.

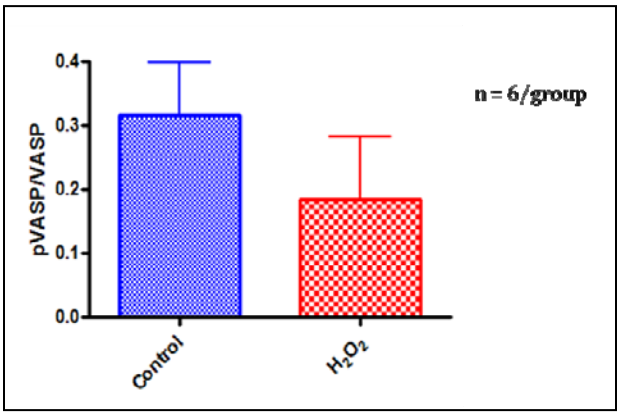


Figure 34: 10 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 does not significantly affect cardiac pVASP protein expression.
Adult male ICR mice hearts were isolated and subjected to 60 minutes of normoxic perfusion or 10 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Western blot densitometry measurements were calculated as a ratio of pVASP to total VASP which is not changed following oxidant stress when compared to respective normoxic perfusion controls. Bar graphs represent the mean ± SEM.

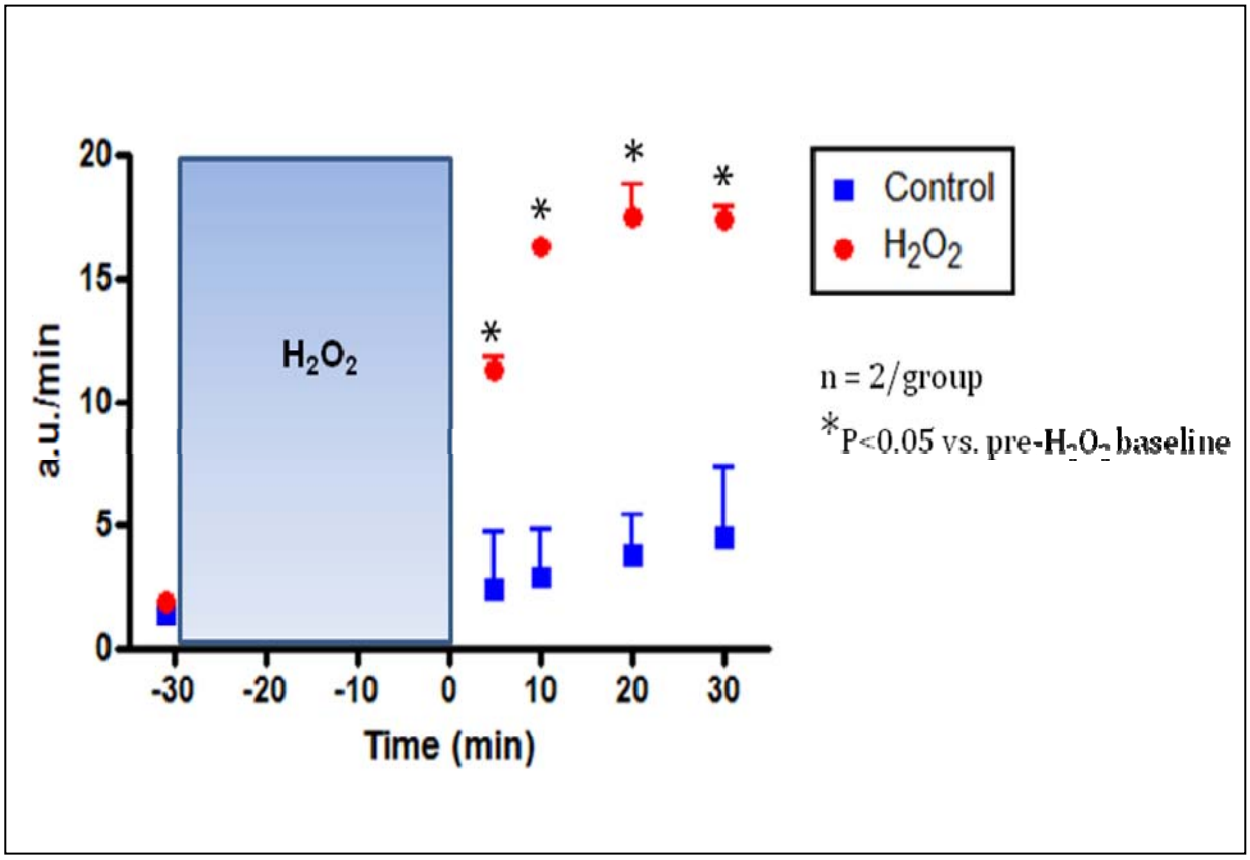


Figure 35: Leakage of intracellular LDH due to 30 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 infusion.
Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion, or 30 minutes perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Leakage of LDH in heart effluent perfusate was measured before and after the 30 minutes of perfusion with H_2O_2. Measurements of LDH activity were done spectrophotometrically using a commercially available enzyme assay kit at 490 nm wavelength (expressed as arbitrary unit, a.u./mL) and normalized against the individual coronary flow rate (mL/min). 30 min of H_2O_2 infusion caused a significant increase in the leakage of LDH. Results are shown as means ± SEM.

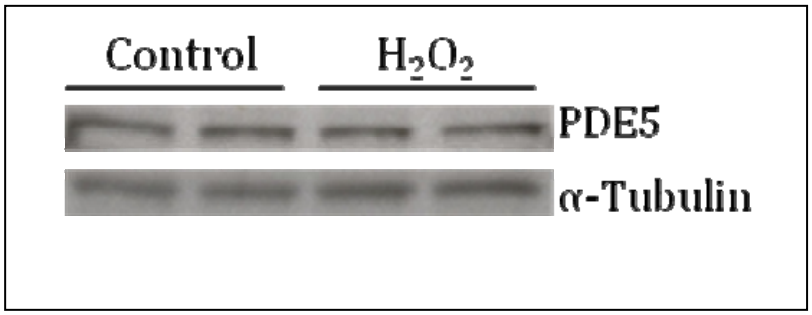


Figure 36: 30 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 infusion does not affect expression of PDE5.
Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion or 30 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by Western blotting with antibody against PDE5 (98 kDa). Blots were reprobed for α-Tubulin (55 kDa) to demonstrate equal loading.

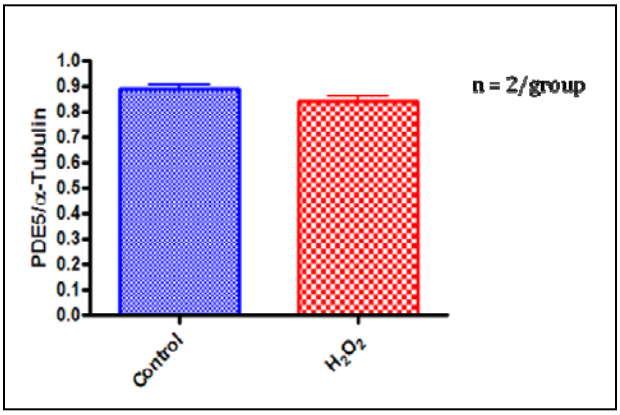


Figure 37: 30 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 infusion does not significantly affect PDE5 protein expression.
Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion or 30 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Western blot densitometry measurements were calculated as a ratio of PDE5 to α-Tubulin which is not changed following oxidant stress when compared to respective normoxic perfusion controls. Bar graphs represent the mean ± SEM.

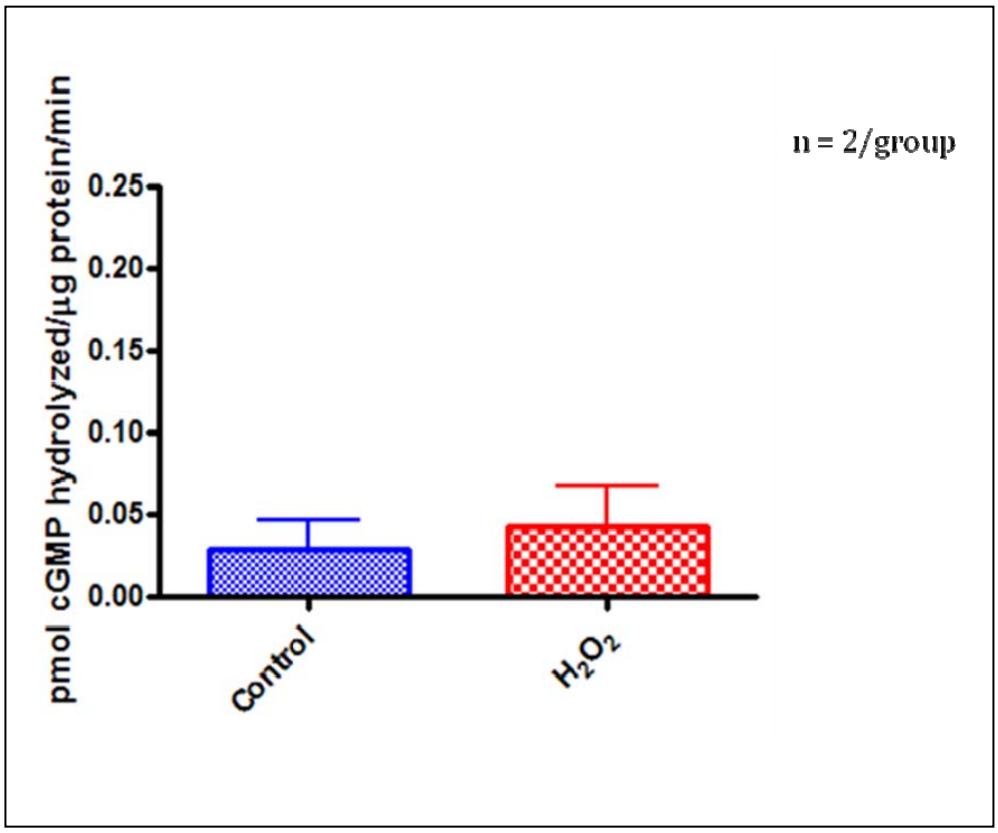


Figure 38: 30 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 does not affect cardiac PDE5 activity.
Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion or 30 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Hearts were harvested for total protein which was assayed for cGMP hydrolytic activity. PDE5-specific activity was measured as the sildenafil-inhibitable fraction of total cGMP hydrolysis, normalized for total micrograms of protein, and is not changed when compared to the respective control normoxic perfusion group. Results are shown as means ± SEM (n=2; read in duplicate).

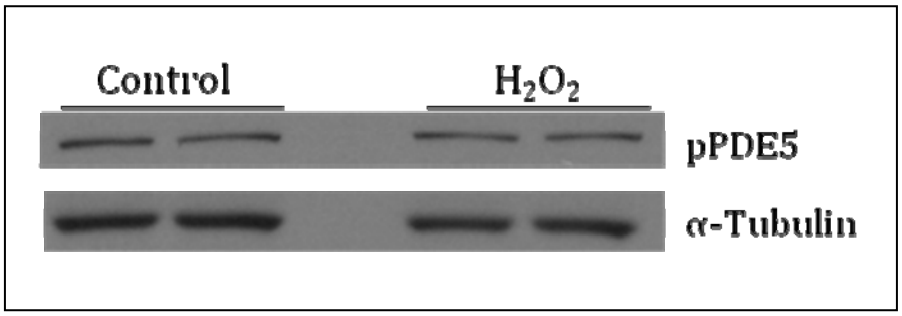


Figure 39: 30 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 does not affect cardiac expression of pPDE5.
Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion or 30 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by western blotting with antibody against pPDE5 (120 kDa). Blots were reprobed for α-Tubulin (55 kDa) to demonstrate equal protein loading.

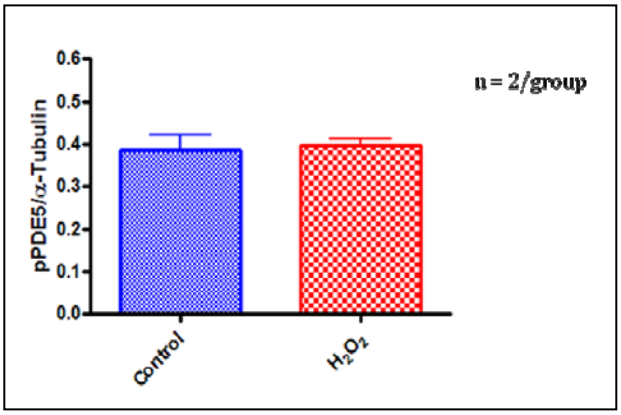


Figure 40: 30 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 does not significantly affect cardiac pPDE5 protein expression.
Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion or 30 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Western blot densitometry measurements were calculated as a ratio of pPDE5 to α-Tubulin which is not changed following oxidant stress when compared to respective normoxic perfusion controls. Bar graphs represent the mean ± SEM.

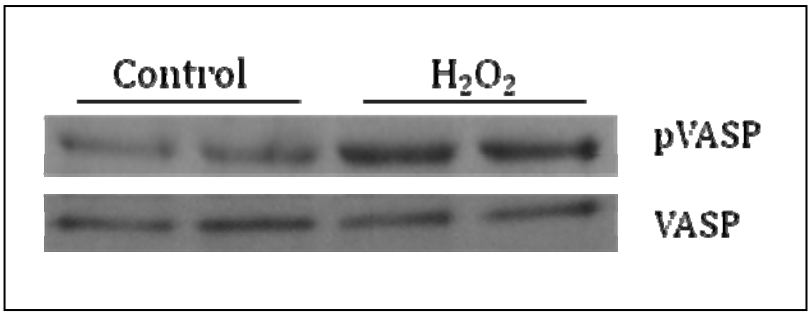


Figure 41: 30 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 infusion upregulates expression of pVASP.
Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion or 30 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Following treatment hearts were harvested and equal amounts of whole heart homogenate protein were resolved by SDS-PAGE and analyzed by Western blotting with antibody against pVASP (48 kDa). Blots were reprobed for total VASP (46 kDa) to ensure that any changes in phosphorylation of VASP were not due to changes in its total protein levels.

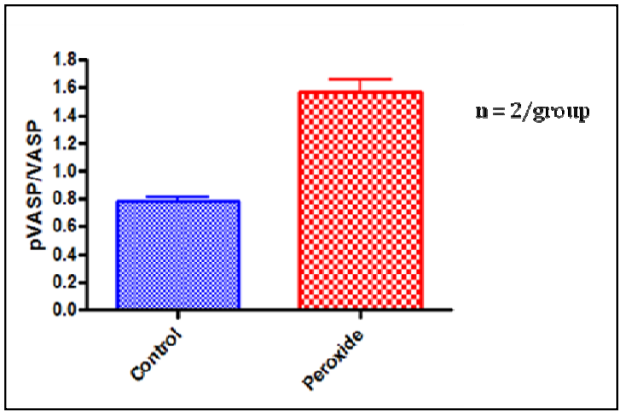


Figure 42: 30 minutes of *ex vivo* oxidant stress with 100 µM H_2O_2 infusion significantly increases cardiac pVASP protein expression.
Adult male ICR mice hearts were isolated and subjected to 90 minutes of normoxic perfusion or 30 minutes of perfusion with 100 µM H_2O_2 followed by 30 minutes washout. Western blot densitometry measurements were calculated as a ratio of pVASP to total VASP which is significantly increased following oxidant stress when compared to respective normoxic perfusion controls. Bar graphs represent the mean ± SEM.

Experimental Protocol	PDE5 Expression	PDE5 Activity	pPDE5 Expression	pVASP Expression
***in vivo* I/R**	↑	↑	—	—
***ex vivo* I/R**	↑	↑	—	↑
10 min. H_2O_2	—	—	—	—
30 min. H_2O_2	—	—	—	↑

Table 1: Summary of experimental results

Discussion

4.1 Rationale of the Present Study

The concept of pharmacological preconditioning with selective PDE5 inhibitors protecting the heart against I/R injury was first introduced by Ockaili et al. in 2002 from our laboratory.[38] In that study and in a series of publications thereafter, our laboratory has shown that this preconditioning-like cardioprotective effect against I/R involved upregulation of eNOS and iNOS, activation of PKC/ERK, opening of mitochondrial K_{ATP} channels, and attenuation of apoptosis by increasing the Bcl2/bax ratio.[38,39,41-43] Still, as diverse as these mechanisms are, none of these previous studies has shown a direct involvement of PDE5 or relate to the known pharmacological action of sildenafil on inhibiting PDE5. The purpose of the current investigation was to identify the role of PDE5 within the heart and specifically its response to cardiac ischemia/reperfusion injury. We postulated that the detrimental events of ischemia and reperfusion lead to a deleterious upregulation of PDE5 which is connected to the production of ROS and contributes to the adverse cardiac stress response that follows myocardial infarction. Of particular interest was defining PDE5 within the signaling mechanisms underlying the already proven cardioproctective action of selective PDE5 inhibitors.

4.2 Salient Findings

The major findings of this study are summarized as follows: 1. *In vivo* regional ischemia and I/R increase the expression and trend to increase activity of PDE5 without affecting PKG activity. 2. *Ex vivo* global ischemia and I/R increase the expression and trend to increase activity of PDE5 as well as enhance PKG activity. 3. 10 minutes of *ex vivo* oxidant stress had little to no effect upon PDE5 expression and activity as well as PKG activity. 4. 30 minutes of *ex vivo* oxidant stress had little to no effect upon PDE5 expression and activity yet raised PKG activity. Taken together, these results provide the first evidence that in acute cardiac ischemia and I/R, PDE5 upregulation may contribute to a deleterious decline in cGMP and cytoprotective PKG signaling, potentially exacerbating dysfunction. Additionally, there appears to be no connection between exposure to ROS and the induction of PDE5. In fact, our data suggest two completely independent mechanisms where PKG is induced by high levels of oxidant stress and PDE5 is induced by the mediators other than ROS resulting from an ischemic event.

While our *in vivo* model shows both ischemia and I/R upregulate PDE5 with downstream pVASP (an indicator of PKG activity) remaining unaltered, our *ex vivo* model contrastingly shows a similar PDE5 upregulation but with a concordant downstream increase in pVASP. This initially may seem paradoxical or contradictory, but the results garnered from treatment with exogenous H_2O_2 lead to a logical explanation. 10 minutes of oxidant stress resulted in no change in either PDE5 or pVASP expression, whereas 30 minutes of oxidant stress still resulted in no change in PDE5 but a dramatic increase in pVASP expression. So, while low and high oxidant stress both had no effect on PDE5, it

is only the high level of ROS that had a significant impact on PKG. Now the process becomes clearer with respect to the ischemia models. *In vivo*, by ligation of the LAD, only approximately 30 to 40% of the cardiomyocytes undergo ischemia. *Ex vivo*, the heart is subjected to global zero-flow ischemia, and by definition 100% of the cardiomyocytes undergo ischemia. So we believe that what is being witnessed within each of the models can be considered under a "low" or "high" oxidant stress paradigm, respectively. The lower number of ischemic cardiomyocytes *in vivo* produce less ROS leading to relatively low oxidant stress, having no effect on PKG. The higher number of ischemic myocytes in the *ex vivo* model produce much more ROS leading to high oxidant stress, which activates PKG. Lastly, while both the *in vivo* and *ex vivo* hearts exhibited the increased PDE5 in response to ischemia and I/R, neither low nor high levels of exogenous oxidant stress had any effect on PDE5. Taken all together, this leads to the aforementioned speculation that the upregulation of PDE5 is likely triggered via other mediators or factors following ischemia and I/R, independent of exposure to the generated ROS (Fig. 43).

Overall a pathogenic PDE5 upregulation during ischemia and I/R may have an important role in facilitating the acute stress response after AMI, as well as adverse remodeling and functional impairment. Combined with data obtained from the previous studies published by our group proving that PDE5 inhibition prior to AMI leads to a dramatic reduction in infarct size in mice,[39] these novel results mechanistically validate the therapeutic strategies to combat this pathogenic PDE5 upregulation with selective PDE5 inhibitors.

4.3 Upregulation of PDE5 triggered by in vivo ischemia and I/R

The presence of PDE5 within the heart has been a contentious issue with many positive and negative findings.[95-100] Recently, our lab was able to detect PDE5 expression in the mouse heart and cardiomyocytes by RT-PCR, immunofluorescent staining, and Western Blotting.[43] Still, despite this discovery combined with other evidence of myocardial PDE5 gene expression,[69,101] its protein synthesis and enzyme activity are rather low compared with other tissues such as lung, and have been traditionally thought to be physiologically insignificant.[99,102] In the current investigation, ICR mice underwent regional AMI by LAD ligation and the hearts were analyzed for PDE5, pPDE5 and pVASP expression as well as PDE5 activity. All three treatment groups (i.e. 30 min ischemia alone, 30 min ischemia plus 12 hours or 24 hours of reperfusion) elicited a significant upregulation of PDE5 protein when compared to their respective control sham operated groups. In addition, we observed this upregulation of PDE5 protein surged immediately following 30 minutes of ischemia, and there was a decrease at 12 hours of reperfusion followed by a subsequent increase at 24 hours of reperfusion (Fig. 12, Fig. 13, Table 1). This overall increase in PDE5 expression is scantily dealt with in the existing literature and the factors leading to its occurrence are both poorly understood and intriguing. As stated previously, we initially hypothesized it may be related to the production of ROS created during ischemia and reperfusion, however our results suggest against this assumption (Section 4.5). Still, ischemia and I/R injury set in motion a great number of signaling cascades that could potentially be responsible for the phenomenon we observed. One compelling possibility is angiotensin II (AngII), which has been shown to rapidly increase

PDE5 mRNA and protein expression as well as PDE5 activity in vascular smooth muscles.[135] AngII is a powerful vasoconstrictor peptide that is proven to be produced during ischemia.[136-138] Furthermore, AngII can be produced endogenously in the heart.[139] Hence it is conceivable that activation of AngII may play a role in upregulation of PDE5 and the resultant antagonization of cGMP signaling following myocardial ischemia. Additionally, PDE5 upregulation could instead be attributed to NP (such as atrial natriuretic peptide [ANP] and B-type natriuretic peptide [BNP]), which are known to be released in response to myocardial ischemia. ANP and BNP levels are raised in patients in the early phase of AMI.[140] This increase in NP would then lead to an increase in pGC activity, raising cGMP levels which then could cause PDE5 upregulation perhaps as a feedback countering mechanism to trigger its breakdown in an attempt to maintain cGMP levels within the physiological normal range in response to the insult of ischemia and I/R. Furthermore, PDE5 has been shown to be phosphorylated in response to ANP, [141] however this was not witnessed in our samples (Fig. 16, Table 1). These increased PDE5 levels may relate to cGMP/PKG effects on transcription and posttranslational activation. Yet, the increase we witnessed in PDE5 protein did not lead to an appreciable difference in downstream PKG signaling, as evidenced by an insignificant change in pVASP protein, whose phosphorylation is a direct product of PKG's kinase activity (Fig. 18, Table 1). The results additionally show that, while not significant, all three treatment groups trend to cause an increase in PDE5 enzymatic activity (Fig. 14, Table 1). Thus, this clear demonstration of PDE5 upregulation following cardiac ischemia and I/R suggests it may contribute to a decline in cGMP and potential disruption of protective signaling in the

heart. For example, PDE5 expression is reportedly increased in human and animal models of right ventricular hypertrophy.[107] This elevation did not translate to enhanced PKG signaling when PDE5 inhibitors were applied. Increased PDE5 expression has also been shown to predispose mice to adverse left ventricle remodeling after AMI.[109]

4.4 Upregulation of PDE5 triggered by ex vivo ischemia and I/R

In the current investigation, the hearts underwent ischemia and reperfusion in isolated perfused Langendorff mode. The rationale for using the isolated heart model was to eliminate all circulating factors that exist *in vivo* and demonstrate the direct effect of ischemia and reperfusion on the expression and activity of PDE5 in the intact heart. The hearts underwent global zero-flow ischemia and were analyzed for PDE5, pPDE5 and pVASP expression as well as PDE5 activity. Both treatment groups (i.e. 30 min ischemia alone, or 30 min ischemia plus 30 minutes of reperfusion), elicited an upregulation of PDE5 protein when compared to their respective control 90 minutes of normoxic perfusion. While both groups elicited an increase, the ischemia alone group yielded the greater upregulation, which agrees with our *in vivo* experiments (Fig. 21, Table 1). Interestingly, ischemia alone also concurrently increases downstream PKG signaling, as evidenced by the significant increase in cardiac pVASP protein expression, which is then partially normalized by subsequent reperfusion (Fig. 26, Table 1). The results additionally show that both groups cause an increase in PDE5 enzymatic activity and suggest that, while not significant, the increase due to I/R is greater than the increase following ischemia alone (Fig. 22, Table 1). At first, we postulated the concordant increase in both the PDE5

levels along with pVASP phosphorylation may be indicative of cGMP levels being regulated by a PKG-dependent activation of PDE5. Specifically, an increase in cGMP levels leads to triggering its breakdown by PDE5. At the same time, the activity of PDE5 is enhanced by PKG-dependent phosphorylation initiated by cGMP-specific binding to allosteric sites on PDE5 in an attempt to maintain cGMP levels within the physiological normal range in response to the insult of ischemia and I/R.[142] Yet this phosphorylation was not witnessed in our samples (Fig. 24, Table 1). As mentioned previously, while we initially hypothesized that ROS production during ischemia and reperfusion may be related to the PDE5 upregulation witnessed, we now believe that this oxidant stress is actually directly affecting PKG independent of PDE5 upregulation. In this model, a great deal of ROS are being produced by the global zero-flow ischemia of the Langendorff, whereby 100% of the cardiomyocytes undergo ischemia. We believe this high level of oxidant stress is directly activating PKG and causing the high levels of pVASP expression (Section 4.5). The partial normalization of pVASP expression following reperfusion is likely a result of washout of the ROS produced during ischemia and upon reperfusion.

4.5 Effect of exposure to exogenous H_2O_2 on PDE5

Heart ischemia is known to induce increased generation of ROS, and subsequent reperfusion can result in toxic ROS overproduction that possibly contributes to irreversible damage of mitochondrial function and consequently impaired recovery of physiological function and cell death.[125-129] In the current investigation, the hearts underwent oxidant stress in isolated perfused Langendorff mode to demonstrate the direct effect of exposure

to ROS on the expression and activity of PDE5 in the intact heart without any circulating confounders that exist *in vivo*. Following 10 minutes of perfusion with the oxygen radical generator H_2O_2 (100 μM) followed by 30 minutes of washout normoxic perfusion, the hearts were analyzed for PDE5, pPDE5 and pVASP expression as well as PDE5 activity. The results showed that 10 minutes of oxidant stress had little effect on the amount of PDE5 or pPDE5 protein when compared to the respective control 60 minutes of normoxic perfusion (Fig. 29, Fig. 31, & Table 1). This same treatment also left downstream PKG signaling unaltered, as evidenced by the unaffected levels of cardiac pVASP protein expression (Fig. 34, Table 1). The results further suggest that a short duration of ROS exposure results in little to no increase in PDE5 enzymatic activity (Fig. 30, Table 1). Some have purported a possible interaction between ROS and PDE5 may potentially lie in the fact that promoter analysis shows both a Sp-1 and an activator protein-1 (AP-1) site in the human PDE5 promoter region.[143] Both of these transcription factors have been shown to be redox sensitive and are claimed to represent potential downstream targets of ROS-mediated signaling on PDE5.[144] However, they are also downstream targets of AngII as well and, as mentioned, this could be a potential mediator of PDE5 upregulation. Farrow et al. have reported that treatment of FPASMCs with exogenous H_2O_2 leads to a significant upregulation of PDE5.[131] Reconciling this with our results may lie in the models themselves. Farrow et al. utilized isolated FPASMCs while we investigated adult whole heart homogenates. Not only were entirely different cell types being studied, but it is possible that ROS have a much different effect on isolated cells when compared to exposure via intra-coronary perfusion of an entire organ. Lu et al. have reported that

myocardial PDE5 expression is increased in CHF along with oxidative stress markers and that this increase is blunted by a SOD mimetic.[110] However the approach in this study also differs greatly from our own. Lu et al. implemented a protocol of chronic TAC induced CHF while we instead looked at the much more acute model of I/R and exogenous administration of H_2O_2. Ultimately, ROS is a very general effecter of many transcription factors and the presence of Sp-1 and AP-1 promoter sites in the PDE5A gene does not implicate a direct connection. Oxidative stress can broadly affect many signaling pathways by altering the expression and modification of many important molecules. A precise molecular mechanism by which oxidative stress can regulate PDE5 expression has not been defined and our results indicate that such a potential link does not sufficiently lead to the induction of PDE5, and this induction instead occurs via other mediating factors independent of ROS.

Further emphasizing this point, additional hearts were subjected to 30 minutes of H_2O_2 (100 μM) perfusion followed by 30 minutes of washout normoxic perfusion, and again analyzed for PDE5, pPDE5 and pVASP expression as well as PDE5 activity. The longer duration of oxidant stress had little effect on the amount of PDE5 or pPDE5 protein when compared to the respective control 90 minutes of normoxic perfusion (Fig. 37, Fig. 40, & Table 1). However, this treatment did lead to an increase in downstream PKG signaling, as evidenced by the significantly higher levels of cardiac pVASP protein expression (Fig. 42, Table 1). The results additionally suggested that a long duration of ROS exposure results in unchanged PDE5 enzymatic activity (Fig. 38, Table 1). This result, given the longer exposure to oxidative stress, again underscores the lack of a

connection between ROS and PDE5. However, there is a growing body of literature showing PKG is regulated by cellular redox status.[145,146] In fact, it was recently shown that oxidation of PKG by exogenous H_2O_2 directly activated PKG and this oxidation-induced activation indicates an alternate mechanism for its regulation independent of the classical activation via cGMP.[147] Thus, it is likely that the observed induction of PKG following 30 minutes of oxidative stress is proceeding through this mechanism and it appears the oxidation-induced activation is time dependent with respect to exposure duration to H_2O_2, given that 10 minutes of oxidative stress produced little change in cardiac pVASP expression.

4.6 Future Studies

More work is needed to better elucidate the pathophysiologic conditions under which PDE5 upregulation occurs as well as the principal mediating factors involved (e.g. by using AngII receptor blockers). Additional investigations are also required to further demonstrate the lack of a relationship between ROS and PDE5. This would be accomplished by monitoring PDE5 and pVASP levels following administration of antioxidants such as N-2-mercaptopropionyl glycine (MPG) to the hearts subjected to ischemia and I/R in the isolated perfused heart model. According to our current theory, PKG activity would be blocked by antioxidants while PDE5 upregulation still present, providing a more definitive illustration of two separate mechanisms at work. Furthermore, it would be highly valuable to reproduce the current results within isolated ventricular cardiomyocytes themselves to demonstrate the cell type specificity.

4.7 Conclusions and Translational Perspectives

It is paramount to recognize that practicing preventative medicine will most effectively control the prevalence and persistence of heart disease and specifically myocardial infarction in our country. However, limitations such as access, availability, education, and other socioeconomic factors of medical care often impair this approach. Therefore, a clinical challenge exists in finding therapeutic strategies to limit and reduce ischemic insult, thus preserving heart function. Novel medical treatments that will effectively limit the extent of tissue damage caused by myocardial infarction are urgently needed. Identifying mechanisms of endogenous cellular protection or tolerance against ischemia is coming to the forefront of research in order to find new methods for treating cardiovascular diseases. This study acknowledges that fact and identifies PDE5 as a valid and novel target to combat the detrimental effects of a heart attack.

Our present studies have provided the first evidence that cardiac PDE5 upregulation, occurring as a result of infarction, is independent of oxidant stress and it may contribute to a deleterious decline in cGMP and a potential disruption of protective signaling in the ischemic heart. The discovery of this pathogenic PDE5 upregulation that occurs following ischemia and I/R further augments and mechanistically validates the argument for therapeutic strategies utilizing selective PDE5 inhibitors in the fight against irreversible heart attack damage. We have witnessed a seemingly ever growing pandemic of heart disease and heart attacks in our country today. Often we are hard pressed to find anyone who has not had a relative or significant other affected by these diseases. Yet the

observations contained within this study help in identifying a novel target for treatment and I am excited that amid growing interest combined with dedication, we may look forward to major strides being taken during our lifetime toward making sure further life is not lost.

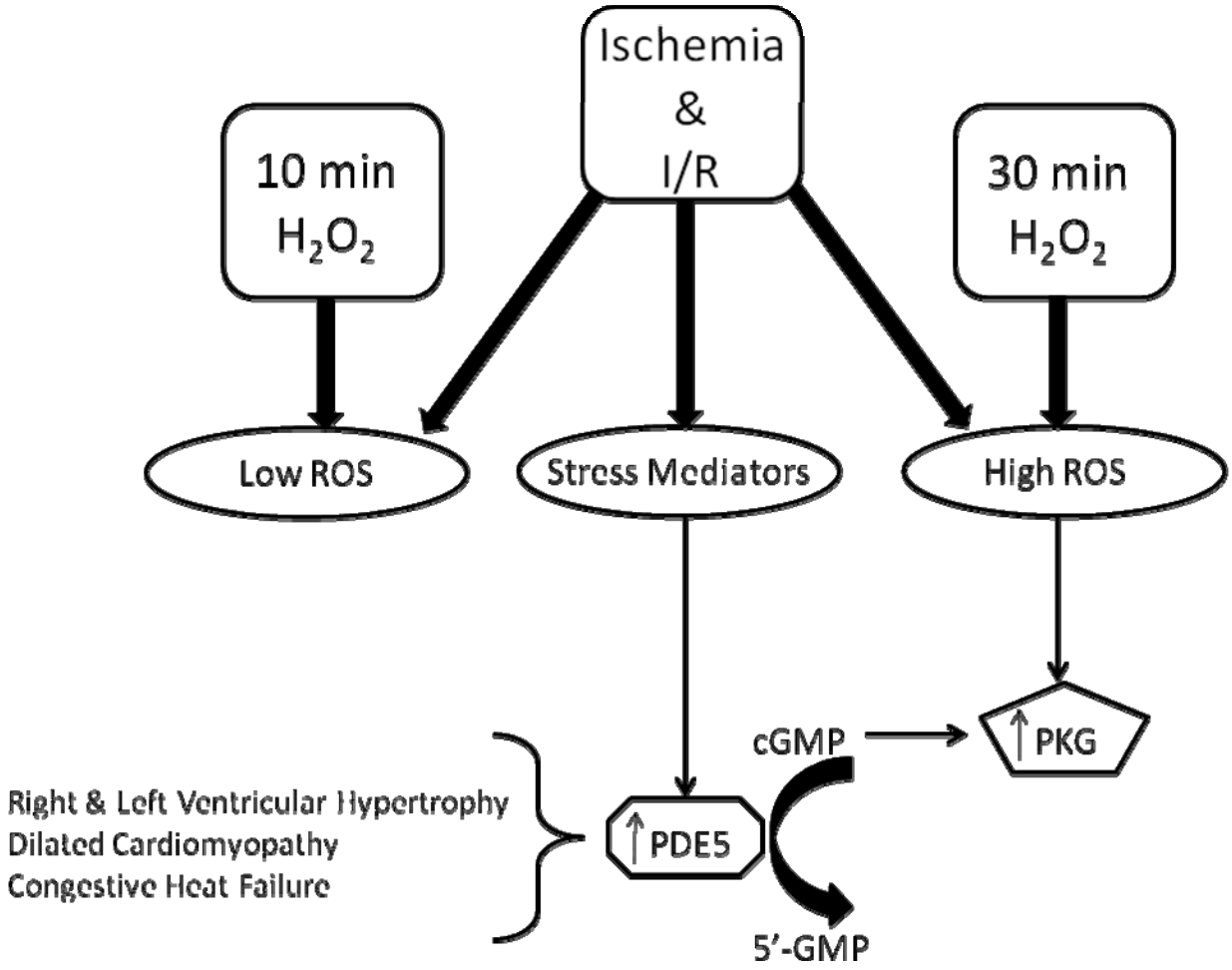


Figure 43: Summary of ROS-independent upregulation of PDE5 in the ischemic heart.

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Vita

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