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Novel Strategies in Cardioprotection against Ischemia/Reperfusion Injury

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NOVEL STRATEGIES IN CARDIOPROTECTION AGAINST

ISCHEMIA/REPERFUSION INJURY

A Dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by

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Table of Contents

<u>List of Tables</u>

Page

List of Figures

Chapter 7:

Chapter 8:

Abstract

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ISCHEMIA/REPERFUSION INJURY

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A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Cell damage represents a major pathomechanism in many diseases of high clinical interest, such as myocardial infarction (MI), where it plays an important role in ischemia-reperfusion (I/R) injury. Considerable progress has been made towards identifying physiological and pharmacological agents that play a key role in myocardial preconditioning against I/R injury and also elucidating the molecular changes leading to such protection.

Second messengers in cellular signaling pathways, such as cGMP have been well implicated as key players in ischemic and pharmacological preconditioning (PC) of the

heart. Phosphodiesterase type 5 (PDE-5) is an enzyme that specifically hydrolyzes cGMP thereby decreasing its tissue concentration. Sildenafil is a potent selective inhibitor of PDE-5 and therefore allows the accumulation of cGMP in several tissues shown to express PDE-5, including pulmonary and coronary arteries. We initially hypothesized that vasodilation induced by sildenafil may release several endogenous mediators including adenosine, bradykinin or nitric oxide (NO), that may trigger a signaling cascade leading to protection against ischemia/reperfusion (I/R) injury. Our results show that sildenafil, at a clinically relevant dose, induced powerful acute and delayed cardioprotection against I/R injury in an *in vivo* rabbit model via opening of mitoK$_{ATP}$ channels. The acute cardioprotective effect of sildenafil was dependent on activation of protein kinase C in rabbits. Moreover, we observed that sildenafil induced delayed PC by NO produced through activation of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) in the mouse heart. The expression of iNOS/eNOS was regulated by ERK phosphorylation and the delayed protection against I/R was blocked by PD98059, a selective ERK inhibitor. Furthermore, sildenafil-induced delayed protection was abolished in the intact heart as well as adult myocytes derived from adenosine A1 receptor knock-out mice suggesting an essential role of A1 receptor in protection. Taken together, these studies suggest that sildenafil is a powerful tool to reduce I/R injury in the animal models. Future clinical studies with relatively safe and effective PDE-5 inhibitors may have an enormous impact on the use of these compounds in reducing I/R injury in the heart and other organs.

CHAPTER 1

INTRODUCTION

1. Background

Determining the mechanism(s) of cardioprotection in diseased hearts is of high significance because the mortality and morbidity of heart disease are increasing around the world, and there is a continuous demand for safe and efficient preventive or therapeutic strategies. Following the development and application of percutaneous transluminal coronary angioplasty (PTCA) [4] and percutaneous transluminal coronary recanalization (PTCR) in the clinical setting, treatment of patients with acute coronary syndrome has made significant progress [5]. As a result, mortality related to acute myocardial infarction has decreased, but the functional recovery of reperfused hearts is unfortunately less than expected, resulting in an increase of patients with chronic ischemic heart failure [6]. As of yet, no sufficiently effective drugs to decrease infarct size and prevent cardiac remodeling in patients with acute myocardial infarction or ischemic heart disease have been identified .

Cardiologists involved in the management of patients with acute coronary syndrome, including severe unstable angina and acute myocardial infarction, occasionally used to report "the cardiac warm-up phenomenon" [7], in which patients with at least one episode of angina showed less severe ischemic damage after subsequent exposure to a

1

longer period of ischemia. In 1986, Murry et al. [1] first documented this phenomenon experimentally and termed it "ischemic preconditioning" (PC). Following this initial report, numerous studies were performed using various tissues and animals (e.g., liver [8], kidney [9], brain [10], and endothelial cells [11]). PC was shown effective against subsequent lethal ischemic insults in all models. More importantly, PC has also been shown to occur in humans by some clinical studies [11]. This realization is highly significant due to the possibility of transferring some or all of the basic research findings from experiments involving PC to the clinical forefront in order to minimize infarct size resulting from ischemic injury in patients with cardiovascular disease. Brief periods of ischemia that cause preconditioning **must** occur prior to sustained ischemia to achieve cardioprotection, but induction of myocardial ischemia is not a practical treatment for patients with ischemic heart disease or those at risk of cardiovascular disease. Therefore, it is important to determine the mechanisms underlying ischemic preconditioning in order to devise new strategies effective against ischemic insults. Identifying some of the triggers, mediators, and effectors of ischemic preconditioning would make it much easier to develop more effective therapeutic interventions for acute coronary syndrome. Accordingly, research in this field has been aiming at recognizing the histological, biochemical, and physiological changes in the myocardium induced by ischemia in order to provide ideas about the cellular mechanisms of ischemic preconditioning. Thus far, intensive research has led to considerable progress in understanding the fundamental nature of ischemic preconditioning and many of the potential pathways involved.

2. Windows of Protection

Soon after the original studies on ischemic PC revealed its potent cardioprotective effect against I/R injury, independent reports from two laboratories in 1993 demonstrated that the cardioprotective effect of ischemic preconditioning on infarct size was still detectable for 24 hr after preconditioning and was associated with increased expression of high molecular weight heat shock proteins (HSPs) [12, 13]. Because Kuzuya et al. [12] also found that the infarct-limiting effect of preconditioning dissipated between 3 and 12 hr after a brief period of ischemia, these two periods of cardioprotection were then referred to as the "first window" and "second window", respectively, which are now known as "early phase (or classical)" and "late phase" preconditioning. Subsequent research on the late phase of cardioprotection afforded by PC also showed that it can persist for up to 72 hr after preconditioning [14]. Thereafter, studies on PC focused on investigation of the subcellular mechanisms, with the aim of devising new methods for application to pharmacological preconditioning and for new clinical therapies to control acute myocardial infarction and the resulting damage to the myocardium.

Interestingly, although different mechanisms may be involved in their mediation of cardioprotection, the "early phase" and "late phase" of PC were reported to share some trigger mechanisms. However, whereas the early phase is dependent on reactions that occur very rapidly, such as direct activation of ion channels or phosphorylation of enzymes and proteins, the late phase involves processes that require a longer period to occur such as

modulation of the genes regulating channel proteins, receptor proteins, enzymes, molecular chaperon proteins, or immune factors. So, in other words, it is quite interesting how these two types of cardioprotection that occur at different time windows might share certain triggers, mediators, and effectors.

For the purpose of the current discussion, we decided to individually describe the cellular mechanism(s) involved in each of the two phases of PC.

3. Early phase PC: Cellular mechanisms

Adenosine

A little over a decade ago, a study showed that inhibition of the adenosine receptor by administration of 8-p-sulfophenyl theophylline (8-SPT) prior to sustained ischemia was able to abolish the protective effect of ischemic preconditioning in a rabbit model [15]. This report suggested that adenosine was a strong candidate as an agent for pharmacological preconditioning and a trigger factor for ischemic preconditioning.

Taken together with the facts that the adenosine A_1 receptor is one of the typical G-protein-coupled receptors (GPCRs) that have seven transmembrane domains, and that the intracellular Ca^{2+} concentration rises soon after the onset of ischemia, it was hypothesized that the subsequent cardioprotective response to an adenosine A_1 receptor-derived stimulus is related to both G-protein signals and Ca^{2+}. As a result of myocardial ischemia, there is

an immediate depletion in the ATP that is available for the cardiac cells [16] and as a consequence adenosine is released [17] to interact with its receptors. In this respect, it was believed that brief episodes of I/R would cause the release of adenosine and allow the interaction with its receptors to "precondition" the myocardium against future ischemic insults. Moreover, intracoronary infusion of adenosine in the isolated rabbit heart [18] or 2-chloro-N^6-cyclopentyladenosine (CCPA) in the isolated mouse heart prior to sustained I/R demonstrated a marked decrease in infarct size when compared to control animals. In addition, intravenous administration of another adenosine agonist, R-phenylisopropyl adenosine (R-PIA) also produced similar results following sustained I/R. Furthermore, in a study performed on transgenic mice, it was shown that over-expression of cardiac A_1 adenosine receptor provided protection against I/R injury. There appears to be a dose dependent manner in which these adenosine receptor agonists operate. Whereas three doses of CCPA significantly reduced infarct size in the rabbit heart [19, 20]; continuous adenosine A_1 receptor activation with a chronic infusion of CCPA causes down-regulation of the signaling cascade and the loss of the protective effect [21]. Despite these observations, the intermittent administration of CCPA 10 day period did in fact confer cardioprotection 48 hours after the last dosing [22].

Protein Kinase C (PKC)

Protein kinase C (PKC) was first described as a calcium-activated, phospholipids-dependent serine/threonine protein kinase. PKC is activated by diacylglycerol (DAG)

hydrolyzed from phosphatidylinositol (PI) by phospholipase C (PLC) under different cell-signaling systems and has been an attractive intracellular receptor for tumor-promotor phorbol esters. Despite the fact that PKC has been implicated as a protein kinase, further molecular studies revealed that it belongs to a family of serine/threonine-specific protein kinases and is involved in diverse cellular functions, including cell proliferation, tumor promotion, differentiation, and apoptotic cell death.

PKC consists of at least 11 isoenzymes with selective tissue distribution, activators, and substrates. Some of the isoenzymes are classified as classical or conventional PKC, which require phospholipids, DAG, or phorbol ester and calcium, and others were classified as novel PKC which share the same requirements with conventional PKC with the exception of calcium. More recently, a different class of PKC emerged and that is the atypical PKC which depends on phospholipid only.

Classical PKCs are activated by DAG and calcium in the presence of phosphatidylserine (PS) and constitutively present in the membrane as follows: (i) DAG and inositol-1,4,5-triphosphate (IP3) are generated from plasma membrane–associated phosphatidylinositol-4,5-diphosphate (PIP2) by the activation of PLC; (ii) Ca^{2+} is released from an intracellular storage pool stimulated by IP3; (iii) Ca^{2+} is bound to the C2 region of PKC and the enzyme is subsequently translocated to the plasma membrane where (4) it is activated by DAG and PS. Phorbol ester, in this model, would mimic the action of DAG, and, by its persistence in the cellular membrane, lead to the long-term activation of PKC. As mentioned, nPKCs do not need Ca^{2+} for activation, but they do require DAG/PS or phorbol ester/PS. These processes are quite rapid, taking place in seconds or minutes, so that a more pronounced

activation of PKC is required for cellular response to growth factors, cytokines, and phorbol ester. Sustained activation of PKC is obtained by the long-term generation of DAG from phosphatidylcholine by phospholipase D (PLD). Phosphatidylcholine is hydrolyzed to choline and phosphatidic acid that is then converted to DAG by phosphomonoesterase, thereby activating PKC. Intriguingly, PLD is activated by PKC activators such as TPA and PKC itself. Furthermore, unsaturated fatty acids, such as oleic, linoleic, linolenic, and arachidonic acid, and lysophosphatidic acid produced from phosphatidylcholine by phospholipase A2, have been shown to enhance the activation of PKC by DAG. Cytokines such as interleukin (IL)-3 and IL-1 induce phosphatidylcholine hydrolysis, but not inositol phospholipids turnover; thus, the signal-induced production of a distinct second messenger or activator may decide which PKC isoform becomes activated or not.

Evidence for a significant role for PKC-α in the prevention of the apoptotic process is increasing. Phosphorylation and activation of PKC- α are blocked by ceramide via activation of okadaic acid–sensitive protein phosphatase (ceramide activated protein phosphatase [CAPP]). Ceramide also inhibits both the translocation of PKC- α from the cytosol to the particulate fraction and activation of PLD, interfering with PKC-mediated activation of PLD. Cell permeable C2-ceramide inhibits activation of PLD and decreases PKC- α activity. PKC- α may prevent apoptosis via activation of Bcl-2, as (1) ceramide-induced apoptosis is prevented by Bcl-2; (2) cPKC-mediated phosphorylation of Bcl-2 at serine 70 functionally suppresses apoptosis; and (3) PKC- α co-localizes with Bcl-2 at the mitochondrial membrane and phosphorylates it, thereby preventing progression of the

apoptotic sequence. PKC-ε is also postulated to function as a suppressor of apoptosis, because Bcl-2 is a substrate of PKC- ε. Overexpression of PKC- ε in human IL-3–dependent TF-1 cells suppresses apoptosis induced by withdrawal of the cytokine via induction of Bcl-2 expression. PKC- ε appears to be inhibited in TNF-α-, Fas-, and ceramide-induced apoptosis, where the ε isoform is displaced from the particulate fraction to the cytosol.

Ytrehus et al. [23] found that inhibition of PKC abolishes the limitation of infarct size by pretreatment with adenosine or by ischemic preconditioning, suggesting that PKC plays a pivotal role in the cardioprotective effect of preconditioning. Because PKC can be activated by either ischemia or by extracellular stimuli such as catecholamines (α_1-adrenoceptor stimulation), lipopolysaccharide (LPS), and PMA [24 and 25], thus leading to cardioprotection [26], it has come to be recognized as a major mediator of ischemic preconditioning.

On the other hand, it has been reported that transient Ca^{2+} overload prior to sustained ischemia also has the same protective effect as ischemic preconditioning [25]. Furthermore, the cardioprotective effect of PKC is abolished by GF109203X, a selective inhibitor of Ca^{2+}-dependent PKC (classical PKC) [27]. Because PKC-α is Ca^{2+}-dependent, these data suggest that triggering of the early phase preconditioning may be closely associated with transient changes of intracellular Ca^{2+} during a brief period of ischemia. While investigations were performed to identify the responsible subtype(s) of PKC in a dog model [28] and a rat model [29], it was found that PKC-α mediates cardioprotection

due to preconditioning in dogs, while other isozymes such as PKC-δ [30, 31 and 32] or PKC-ε [33] have been detected in smaller animals.

Ischemic PC, as initiated by brief separate episodes of I/R, causes the release of endogenous compounds such as adenosine, bradykinin, catecholomines, opioids which in turn instigate signaling cascades leading to activation of PKC. PKC can also be activated by reactive oxygen species which are known to be generated during short episodes of ischemia especially at the onset of reperfusion. PKC potentially activates certain transcription factors which play an important role in the synthesis of several proteins including indcible nitric oxide synthase (iNOS), antioxidants, Bcl-2 and stress proteins. iNOS generates NO and hence leads to the formation of cGMP from guanylyl cyclase (GC), and cGMP in turn may activate protein kinase G (PKG) leading to opening of mitochondrial K_{ATP} channels. By preventing calcium overload and preservation of ATP, opening of these channels causes cardioprotection against I/R injury.

Administration of PKC agonists in the isolated rat heart resulted in myocardial preconditioning [34]. However, PKC inhibitors given at the end of the ischemic PC period in vivo [35] blocked the protection of PC. This reveals that there is a critical period at which PKC exerts its cardioprotective effect during sustained ischemia but not the PC stage [36].

Since pertussis toxin pretreatment blocked the protective effect of PC [37], it was suggested that the A_1 adenosine receptor coupled to the G-protein system is a key player in inducing cardioprotection by PC. Other receptors including muscarinic M_2 [38], adrenergic α1 [39], and bradykinin B_2 [40] are also coupled to the G-protein system and

were shown to activate PKC and mimic the effect of ischemic PC [41]. A1 adenosine receptor-G protein- coupling activates phospholipase C which degrades phospholipids of the membrane to DAG. As a result, DAG activates PKC and causes its translocation from the cytosol to the membrane by the microtubules [42]. In deed, blocking this activity with colchicine abolished the effect of ischemic PC, demonstrating the importance of PKC translocation in PC [35]. Other studies also showed that it is not essential for PKC to be continuously activated in order to cause cardioprotection by PC; however, the phosphorylation of certain downstream proteins by PKC prior to sustained ischemia is necessary for achieving an infarct-sparing effect with PC.

ATP-sensitive potassium channels (KATP channels)

Research involving ATP-sensitive potassium channels (KATP channels) has a longer history than studies on ischemic preconditioning, since their existence in the myocardium was first reported in 1983 [43].

The KATP channel exists in the membrane and is modulated by Mg and ATP [44]. KATP channels were first identified by cardiovascular physiology studies as causing vascular smooth muscle relaxation in either large or small arteries or as having a negative inotropic effect on cardiac myocytes [45]. In addition, activation of these channels seems to occur as a response to decreased intracellular ATP levels, which is normally achieved within a few minutes after the onset of ischemia. Because KATP channels act as an inward rectifier [44] when activated, these effects could be caused by an increase of the depolarization

threshold that reduces excitation of either vascular smooth muscle or cardiac myocytes followed by vasodilation and shortening of the action potential duration, respectively, which finally lead to intracellular Ca^{2+} unloading [45] and, as a result, reduced metabolic demand. Since these phenomena resemble the acute cardiac responses and cardioprotection afforded by ischemic preconditioning, it has been postulated that KATP channels might be one of the critical effectors of both ischemic and pharmacological preconditioning [46]. Pharmacological preconditioning with KATP channel openers was first demonstrated in 1992 [47], and it was thought to be an intervention downstream of the A1 adenosine receptor [48].

An early study in 1991 [49] demonstrated that not only the cell membrane but also the inner mitochondrial membrane possessed ATP-sensitive inward rectifier activity, and therefore suggested the existence of "mitochondrial KATP channels" as opposed to "sarcolemmal KATP channels". After a long conception that both Ca^{2+} unloading and preservation of ATP were related to sarcolemmal KATP channels, it was found that both processes are actually mediated by mitochondrial KATP channels [50]. As a result, numerous studies on the cardioprotective effect of mitoKATP channel opening have been conducted, including many from our laboratory demonstrating the role of mitoKATP channel-opening in acute as well as delayed PC using several pharmacological agents. Unfortunately, despite all the positive results of PC associated with the opening of these channels, these so called "mitochondrial KATP channels" have still not been cloned after more than 10 years of investigation in spite of dramatic progress in the cloning of many mitochondrial proteins. Additionally, although some in vitro studies have shown a potent

effect of putative modulators of mitochondrial KATP channels [51] (diazoxide as an opener of KATP channels and 5-hydroxydecanoate (5-HD) as an inhibitor which are almost the only tools available to test the function of these channels), both drugs also have other important direct effects on cellular respiratory metabolism. Some recent studies using other models have failed to show complete modulation of the cardioprotective effect of ischemic preconditioning by these two drugs [52 and 53]. However, Pain et al. [54] have reported that reactive oxygen species (ROS), which are transiently generated by opening of the mitochondrial KATP channels, activate downstream cascades that confer cardioprotection. It is not clear why these differences exist, but some investigators focusing on mitochondrial KATP channels have suggested that the contribution of sarcolemmal KATP channels to cardioprotection may be more important in the beating hearts and less important under nonbeating experimental conditions (i.e., in vitro). Although many ongoing studies are providing some possible features or characteristics of mitochondrial KATP channels, the actual cardioprotective role of these channels needs to be investigated further in order to establish the cause-and-effect relationship between mitoKATP channel-opening and cardioprotection with PC.

p38MAPK or PI3-K and their downstream cascades

There are over 1500 protein kinases identified so far among all different subclasses. Their main function is to transfer a terminal phosphate group from ATP or GTP to an amino acid (AA) residue; serine, threonine, or tyrosine. Different groups involve protein

kinases that specifically phosphorylate serine or threonine, referred to as ser/thr protein kinases, or tyrosine protein kinases that would specifically phosphorylate tyrosine. Another group of protein kinases that phosphorylates all 3 AA's is a family known as mitogen activated protein kinases (MAPK). This family is yet the least understood. The major role that these protein kinases play is intracellular. When cells receive external signals initiated by several factors such as growth factors, cytokines, pharmacological agents or stress, intracellular pathways are activated which rapidly alter patterns of gene expression. This is the essence of cell signaling and the resulting cellular response to various types of stimuli. A certain extracellular stimulus would potentially activate a specific receptor at the level of the plasma membrane leading to the generation of second messengers within the cell. These chemicals and the extent to which they are expressed may alter the activity of certain protein kinases thus affecting the phosphorylation states of intracellular proteins and ultimately regulating their function. All these changes caused by the various signaling pathways eventually lead to specific physiological responses.

Currently, there are four MAP kinase cascades that are differentially activated depending on stimulus type: the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) 1/2 pathway, the c-Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, the p38 pathway and the big mitogen-activated protein kinase BMK/ERK 5 pathway. Activation of the MAP kinase pathways leads ultimately to the phosphorylation of transcription factors bound to their regulatory elements in the promoters of target genes. Phosphorylation of these transcription factors is crucial for gene activation. The use of relatively specific MAP kinase and other inhibitors has allowed

elaborate investigation of the signaling pathways and/or mechanisms involved in phosphorylation of certain proteins in response to specific stimuli. The p38 pathway is inhibited by SB203580, which specifically binds to and inhibits p38 MAP kinase itself, rather than inhibiting its activation as in the case of some other MAPK's.

Within the past decade, it was proposed and demonstrated that several cellular stresses activated the p38 MAPK pathway. At least four isoforms of p38 were shown to exist, they are termed p38α, β, γ, and δ. There are also several protein kinases downstream of p38 that are activated following its phosphorylation. The role of p38 MAPK signaling in cellular responses is diverse, and has in fact given rise to controversy concerning its role in preconditioning of the myocardium depending on several factors such as cell type and stimulus.

Despite the controversy concerning the physiological significance of p38 MAP kinase in the heart, it has been demonstrated that p38 is stimulated by I/R. Recent studies suggest that increased phosphorylation of p38 MAPK occurred in the preconditioned heart, which was completely abolished by SB203580, a selective inhibitor of p38 MAPK. It has also been demonstrated that increased p38 phosphorylation plays a pivotal role in several cardiac preconditioning approaches whether physiological or pharmacological in nature. As a result, the role of mitogen-activated protein kinases (MAPKs), which form part of the second messenger systems that mediate extracellular stimuli, in the cardioprotective effect of ischemic preconditioning has been further studied.

Not too long ago, a study in rabbits reported that ischemic preconditioning caused an increase of p38MAPK activation during sustained ischemia which was correlated with the cardioprotection provided by preconditioning [55]. However, p38MAPK was not activated during ischemia in the control group, while contradictory results were obtained a few years later by *in vivo* [56] and *in vitro* [57] studies that suggested p38MAPK activation could in fact promote ischemic damage. Treatment with anisomycin, a potent p38MAPK (and JNK) activator, just before ischemia could attenuate ischemic injury, with only the first peak of p38MAPK activation persisting and the second being abolished. This observation led to the hypothesis that p38MAPK activation has opposing effects; i.e., transient activation prevents ischemic injury, whereas continuous activation intensifies it.

Furthermore, MAPKAPK-2 and HSP27 (one of the small heat shock proteins that acts as a molecular chaperone), which are downstream of p38MAPK, are activated in the preconditioned myocardium at the onset of sustained ischemia. Interestingly, MAPKAPK-2 subsequently activates Akt, which lies downstream of PI3-K [58], to antagonize apoptotic signals. PI3-K can be activated by PC [59] and is also reported to cause an increase of nitric oxide (NO) production and the downstream activation of PKC [59]. On the other hand, HSP27 binds to the z-bands of myofibrils to prevent ischemic degradation of myofilaments [60] or prevents the interaction of Apaf-1 with procaspase-9 through binding with cytochrome *c* [61] which is released from the mitochondria and enhances this interaction [62].

Bradykinin and opioids

Several studies have implicated bradykinin [63] as a candidate trigger for preconditioning. The cardioprotective effect of bradykinin has been intensively investigated [64], and it is known that preconditioned hearts show an increase of the interstitial bradykinin level [65]. The downstream effectors are reported to be an increase of nitric oxide production [66] or opening of KATP channels [67] after activation of the bradykinin B_2 receptor.

While bradykinin is considered to be an endogenous trigger of preconditioning, as mentioned above, activation of the δ-opioid receptor by opioid peptides has also been investigated as a potent exogenous trigger of preconditioning in isolated human cardiac tissue [68]. Many reports have suggested that KATP channels are the downstream mechanism of cardioprotection, but the relative contribution of either sarcolemmal [69] or mitochondrial [70] KATP channels to the triggering phase remains unclear. It has also been shown that by selectively activating the δ-opioid receptor with DPDPE, a selective agonist, the survival time in mice increased under hypoxic environments. Gross et al also demonstrated that ischemic PC is mediated by activation of the δ-opioid receptor, and by employing BNTX, the δ-opioid receptor antagonist, the infarct-limiting effect of PC was abolished.

4. Late phase PC: Cellular mechanisms

The late phase of preconditioning, which was initially reported as the "second window of preconditioning" [12 and 13] in 1993, occurs about 24 hr (the actual time varies among species and experimental models) after transient preconditioning and lasts for much longer than the early phase, up to 72 hr post PC.

As previously described, the late phase of ischemic preconditioning has essentially different signaling mechanisms from the early phase (classical ischemic preconditioning) such that it involves processes that require a longer time to occur. Recent studies have revealed that these two types of preconditioning share some triggering mechanisms, while certain mediators and effectors are reported to be specific for the late phase of preconditioning. In this part of our discussion, we will focus on the various mediators or effectors involved in late preconditioning.

Adenosine

The adenosine-triggered cardioprotection that appears after 24 to 72 h is reported to have some unique characteristics [14]. Adenosine-triggered cardioprotection involves both adenosine A_1 and A_3 receptors, and it only reduces the infarct size rather than having an influence on stunning or arrhythmias. Initial transient activation of adenosine A_1 receptors causes activation of subcellular signaling pathways, such as p38MAPK or HSP27 [71], and increases the synthesis of manganese superoxide dismutase (Mn–SOD) [72], but the role of A_3 receptors remains not fully understood.

Nitric oxide (NO)

Nitric oxide (NO) was first identified as endothelium-derived relaxation factor (EDRF). In the cardiovascular system, it was shown to quite a similar role to adenosine such as having negative inotropic effects, vasodilatory effects, and inhibition of cytokine production as well as platelet aggregation. All of these actions are mediated through cyclic GMP as a second messenger [73]. It has also been suggested that an increase of NO prior to sustained ischemia plays a part in either ischemic or pharmacological preconditioning [74], indicating that NO could act as either a trigger or an effector of preconditioning which also resembles the role of adenosine. Furthermore, in contrast with the established role of NO in the triggering of late phase cardioprotection, whether NO can trigger the early phase of cardioprotection remains controversial. NO is reported to share downstream pathways with various other triggers [such as acetylcholine (Ach), bradykinin, opioids, and phenylephrine), involving PKC, KATP channels, and the generation of reactive oxygen species (ROS) in the triggering phase, but its other downstream effects are unknown. Some studies [73] reported that PKC activates NF-κB after several hours which then causes increased expression of inducible NO synthase (iNOS) after 4 to 8 h.

Although we have mentioned a direct cardioprotective effect of NO, it also forms peroxynitrite ($ONOO^-$) and promptly loses its bioactivity as a stimulator of cyclic GMP in the presence of high levels of free radicals. It remains unclear whether peroxynitrite is beneficial or harmful to the myocardium because it has been reported to damage vascular endothelial cells [75], whereas another study showed that formation of $ONOO^-$ is required

for NO to act as a trigger of preconditioning [76]. So, in this respect, it is believed that the levels of NO produced are critical in terms of NO being harmful or beneficial. NO, in large amounts, is highly toxic for cells and eventually causes cell death. On the other hand, if "optimal" concentrations of NO were produced at the right time, NO would activate GC and lead to the formation of cGMP which in turn activates PKG and results in cardioprotection through opening of mitoKATP channels. In addition, accumulation of cGMP, especially in vascular smooth muscle cells, causes smooth muscle relaxation and hence vasodilation. This vasodilatory effect is believed to potentially cause the release of certain endogenous mediators of PC, such as adenosine, bradykinin and acetylcholine. Furthermore, some studies using mice with gene targeting have indicated that endothelial NOS (eNOS) is also responsible for cardioprotection [79]. Again, regardless what the source of NO is, whether it is nNOS, iNOS or eNOS, it could probably be concluded that the overall effect of NO, under optimal doses, on the cardiovascular system is beneficial, although the results might vary among species and experimental models.

Heat shock proteins (HSPs)

Intensive research has been conducted to examine the role of HSPs in the cardioprotection induced by ischemic PC. It has been reported that the expression of HSP70/72, a high molecular weight heat shock protein, peaks at 24 to 48 h after

preconditioning ischemia [78]. The main known fuction of HSP 70/72 is its action as a molecular chaperone; however, its cardioprotective effect may be related to other factors induced by HSPs as previously described for HSP27. Inhibition of PKC, by chelerythrine, prior to preconditioning completely abrogated the induction of these HSPs [79], suggesting that PKC also mediates cardioprotection through the generation of HSPs.

On the other hand, ischemic preconditioning also activates Mn–SOD, and the peak level is seen at 24 h after preconditioning [79]. Because increased expression of Mn–SOD has been detected in a model of HSP72 overexpression [78], it may also be sequentially involved in ischemic or heat stress-induced preconditioning. As a result, some studies were performed to test the effect of exogenous Mn–SOD [80], and they showed that this approach fails to protect the myocardium against ischemic damage or reperfusion injury. This suggests that free radicals may play different roles during the process of ischemia and reperfusion injury depending on the site of their generation, i.e., inside or outside cardiac myocytes.

Arachidonic acid

The most recent studies on the downstream pathways have addressed cardioprotection mediated by the induction of enzymes in the arachidonic acid cascade, mainly cyclooxygenase-2 (COX-2) [81]. iNOS and its product NO may activate COX-2 in

the late phase of cardioprotection [82], but the contribution of its products is currently unknown. These findings are interesting with respect to possible links with further downstream internal mediators that are believed to be the final effectors of preconditioning, as well as the concept that the immune response may be a direct effector of the cardioprotection afforded by preconditioning, and are also encouraging with regard to clinical application.

All the aforementioned mediators of PC are summarized in Figure 1 to illustrate their interaction at the subcellular level in order to bring about the whole concept of myocardial PC.

Figure 1

Concept of Myocardial Preconditioning

Endotoxin **Drugs** **Brief Episodes of Ischemia** **Hyperthermia** **Hypoxia**

ROS, Nitric Oxide, G protein-coupled receptors (adenosine, adrenergic, bradykinin, opioid)

During Lethal Ischemia Ca^{2+} influx ↓, ATP ↑

K_{ATP}↑ ⟸ **PKC, MAPKs, TyrK**   **Early Phase of Protection** (Few minuets to 1 hour)

Phosphorylation

Activation of Transcription Factors (HIF-1, AP-1, NFkB, HSF)

Increase in Transcription of Specific Genes

During Lethal Ischemia Ca^{2+} influx ↓, ATP ↑, Free radicals ↓

Late Phase of Protection (24 to 72 hours)

de novo **Synthesis of Protective Proteins** (iNOS, HO-1, MnSOD, Catalase, HSPs)

Concept of myocardial preconditioning

5. Pharmacological PC

After having discussed many of the mechanisms identified thus far in ischemic PC, and after mentioning more than once that inducing brief episodes of ischemia is not a practical approach for management of patients with cardiovascular disease, we finally reach the major goal behind all these studies. Scientists in this field always desired to devise or discover chemicals or pharmacological agents that are able to target one or more of the signaling molecules implicated in ischemic PC in order to achieve similar cardioprotective results bypassing the ischemic stimulus. So far, among the many candidates suggested by experimental studies, there are two agents that have also been shown to minimize damage due to reperfusion injury in the clinical setting: adenosine and the K_{ATP} channel opener nicorandil.

Adenosine

In a clinical trial [83], patients with acute anterior myocardial infarction who underwent continuous intravenous infusion of adenosine together with PTCR had smaller infarcts and better functional recovery than those without adenosine infusion. In this study; however, no cardiac function data were correlate with the observed reduction in infarct size. Therefore, more studies should be conducted in order to better evaluate the effect of

adenosine or ATP administration in the clinical setting not only on infarct size reduction, but also functional recovery by employing angiography.

KATP channel openers

Some experimental [84] and clinical trials using either intravenous [85] or intracoronary [86] administration have shown that the no-reflow phenomenon following reperfusion can be prevented after successful PTCA, together with a reduced infarct size and better recovery from hypokinesis, by continuous infusion of a KATP channel opener (nicorandil) just after the procedure. Eventhough the no-reflow phenomenon has been proposed to be caused by arterial vasospasm [104], the exact mechanism by which nicorandil improves it remains unclear. Furthermore, the component of the KATP channel (mitochondrial or sarcolemmal) involved in this action of nicorandil is also controversial because many reports have confirmed that nicorandil has a stronger effect (10- to 100-fold) on the mitochondrial KATP channel than on the sarcolemmal KATP channel in vitro [87].

Over the past 30 years, enormous progress has been made in our understanding of the basic biochemical and molecular mechanisms that underlie the development of lethal ischemia-reperfusion injury and the protective actions of therapeutic interventions. While adenosine is known to cause preconditioning and protection of the heart under these circumstances, it has been determined that the clinical effects are not as promising as those initially reported by experimental studies. Over 3 years ago, we undertook a series of

novel investigations to determine the role of phosphodiesterase-5 (PDE-5) inhibitors in cardioprotection. These drugs are a novel class of vasoactive agents that have been developed for treatment of erectile dysfunction in men. Their mechanism of action involves active inhibition of PDE-5 enzyme resulting in accumulation of guanosine monophosphate (cGMP) levels and smooth muscle relaxation in the penis. Our initial hypothesis was that the vasodilatory action of these drugs may cause the release of certain endogenous compounds implicated in PC, such as adenosine, bradykinin, or acetylcholine. These mediators could potentially trigger signaling pathways leading to cardioprotection.

Based on the intriguing hypothesis, we designed and performed the following investigations:

Aim #1) to investigate whether sildenafil could confer cardioprotection against I/R injury in a manner similar to ischemic PC in the rabbit model of regional myocardial infarction;

Aim #2) to demonstrate the behaviour of PKC isozymes after sildenafil treatment and their respective roles in cardioprotection with sildenafil in an *in vivo* rabbit model;

Aim #3) To show that sildenafil could confer PC-like effects not only in the adult rabbit model, but also in infant rabbits;

Aim #4) to examine the role of iNOS/eNOS proteins in the infarct-limiting effect of sildenafil in a mouse model of global I/R;

Aim #5) to elucidate the signaling pathways involving activation of p38/ERK with sildenafil in an isolated mouse heart model;

Aim #6) to test the hypothesis that the adenosine A1 receptor plays a role in cardioprotection with sildenafil using A1 receptor knock-out mice.

Aims 1 to 4 represent published papers in peer reviewed journals. The work on Aims 5 and 6 has been completed and will be submitted for publication in the near future.

CHAPTER 2

Sildenafil (Viagra) induces powerful cardioprotective effect via opening of mitochondrial K_{ATP} channels in rabbits

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Summary: Sildenafil citrate (Viagra) is the pharmacologic agent used for treatment of erectile dysfunction in men. Since this drug has vasodilatory effect, we hypothesized that such an action may induce preconditioning-like cardioprotective effect via opening of mitoK_{ATP} channels. Rabbits were treated with sildenafil citrate (0.7 mg/kg, iv) either 30 min (acute phase) or 24 hrs (delayed phase) prior to 30 min of ischemia and 3 hrs of

reperfusion. MitoK$_{ATP}$ channel blocker, 5-hydroxydecanoate (5-HD, 5 mg/kg, iv) was given 10 min before ischemia/reperfusion. Infarct size was measured by tetrazolium staining. Sildenafil caused reduction in arterial blood pressure within 2 minutes of treatment, which returned to nearly baseline levels 3 minutes later. The infarct size (% of risk area, mean±SE) reduced from 33.8±1.7 in control rabbits to 10.8±0.9 during acute phase (68% reduction, $p < 0.05$) and 19.9±2.0 during delayed phase (41% reduction, $p < 0.05$). Similar acute and delayed cardioprotective effect was observed when sildenafil was administered orally. Systemic hemodynamics also decreased after oral administration of the drug. However, these changes were mild and occurred slowly. 5-HD abolished protection with increase in infarct size to 35.6±0.4 and 36.8± 1.6 during acute and delayed phase respectively ($p < 0.05$). For the first time, we demonstrate that sildenafil induces acute and delayed protective effect against ischemia/reperfusion injury which is mediated by opening of mitoK$_{ATP}$ channels.

Keywords: K$_{ATP}$ channel, viagra, ischemia/reperfusion, infarction

INTRODUCTION

Sildenafil citrate (Viagra) is the first oral agent approved for treatment of erectile dysfunction in men (5,11). It is a selective inhibitor of phosphodiesterase-5 (PDE-5), an enzyme that catalyzes the breakdown of a potent smooth muscle relaxing agent cyclic guanosine monophosphate (cGMP). Sildenfil has been shown to enhance nitric oxide

(NO)-driven cGMP accumulation in the corpus cavernosum of rabbits without affecting cAMP formation. In the absence of NO drive, sildenafil had no functional effect on the human and rabbit isolated corpus cavernosum, but potentiated the relaxant effects of NO on these tissues (20). Also, it has been shown that sildenafil causes mild to moderate decreases in systolic and diastolic pressure because of the inhibition of PDE-5 in smooth muscles in the vascular bed (12). In the present studies, we hypothesized that such a mild vasodilatory effect of the sildenafil in coronary vasculature could potentially release agents such as adenosine, bradykinin or NO that may trigger preconditioning-like effect in the heart. Since opening of the mitochondrial K_{ATP} (mitoK_{ATP}) channel mediates the cardioprotective effect of preconditioning induced by adenosine (1,2,4,6) or sublethal ischemia (3), we further hypothesized that these channels could potentially be involved in the cardioprotective effect of sildenafil. Accordingly, the goals of the present study were: (a) to show that the sildenafil induces both acute and delayed protection against ischemia/reperfusion injury *in vivo* and (b) to demonstrate if the protective effect of this drug is blocked by 5-hydroxydecanoate (5-HD), a selective blocker of mitoK_{ATP} channel (14). Using our *in situ* rabbit model of myocardial infarction, for the first time, we demonstrate that sildenafil induced both acute and delayed cardioprotective effects, which is dependent on the opening of mitoK_{ATP} channel.

MATERIALS AND METHODS

Animals

Male New Zealand White rabbits (2.8 to 3.3 kg) were used for the studies. The care and use of the animals were conducted in accordance with the guidelines of the Committee on Animals of Virginia Commonwealth University and the National Institute of Health (NIH) "Guide for the Care and Use of Laboratory Animals" [DHHS Publication No. (NIH) 80-23, Revised, Office of Science and Health Reports, Bethesda, MD 20205].

Surgical procedure: Infarction protocol

The rabbits were anesthesized with an intramuscular injection of ketamine HCl (35 mg/kg) and xylazine (5 mg/kg). Subsequent doses of ketamine-xylazine (10 mg/kg and 2 mg/kg, respectively) were administered during the experiment as needed to maintain surgical anesthesia. Atropine was administered along with the anesthetic in order to keep the heart rate elevated especially during the surgery protocol. The body temperature was monitored and maintained at 38°C throughout the experimental protocol. The neck was opened with a ventral midline incision and tracheotomy performed followed by intubation. The animal was then mechanically ventilated on a positive pressure ventilator using compressed room air at 30-35 cycles/min with a tidal volume of approximately 15 ml. Ventilator setting and pO_2 were adjusted as needed to maintain the arterial blood gas parameters within the

physiological range. The blood gases and pH were measured 12 times for all the groups during the infarction protocol. The arterial blood gases and pH values ranged between 7.20 and 7.50 with pCO_2 maintained between 20 and 50 mmHg and the HCO_3 level ranging between 15.0 and 28.0 mg/L. The pO_2 ranged between 60 and 150 mmHg with the saturation constantly kept above 90%. The jugular vein was cannulated with a polyethylene (PE) catheter for continuous infusion of 0.9 % saline solution. The carotid artery likewise was dissected and cannulated with a PE catheter for blood sampling and continuous arterial pressure monitoring. Electrocardiographic leads were attached to subcutaneous electrodes to monitor either limb lead II or lead III.

Following stabilization of the hemodynamics, a left thoracotomy was performed through the fourth intercostal space and the pericardium opened to expose the heart. A 5-0 silk suture with atraumatic needle was then passed around the left anterior descending artery (LAD) midway between the atrioventricular groove and the apex. The ends of the tie were then threaded through a small vinyl tube to form a snare. To induce infarction, the LAD was occluded for 30 min by pulling the snare and then fixing it in place by clamping the vinyl tube with a hemostat. A bolus of heparin sodium 500 IU was given immediately before coronary occlusion for prophylaxis against thrombus formation around the snare. Myocardial ischemia was confirmed visually *in situ* by regional cyanosis, ST elevation/depression or T wave inversion on electrocardiogram, hypokinetic/dyskinetic movement of the myocardium, and relative hypotension. After 30 min of ischemia, the snare was released and the heart was allowed to reperfuse for 180 min. This was readily

confirmed by hyperemia over the surface of the previously ischemic-cyanotic segment. The thoracic cavity was covered with the saline-soaked gauze to prevent the heart from drying.

Measurement of Infarct Size

Following completion of ischemia/reperfusion protocol, 500 units of heparin was injected and the heart was removed quickly and mounted on a Langendorff apparatus. The coronary arteries were perfused with 0.9% saline containing 2.5 mM $CaCl_2$. After the blood was washed out, the ligation around the coronary artery was re-tightened and approximately 2 ml of 10 % Evan's blue dye injected as a bolus into the aorta until most of the heart turned blue. The hearts were then perfused with saline to washout the excess Evan's blue, removed from the Langendorff apparatus, frozen and cut into 4 to 6 transverse slices from apex to base of equal thickness ($\approx$1 mm). The slices were then incubated in 1 % TTC solution in isotonic pH 7.4 phosphate buffer at 37^0C for 20 minutes. TTC reacts with NADH in the presence of dehydrogenase enzymes causing the cells viable to stain with a deep red color. Red-stained viable tissue was easily distinguished from the infarcted gray or white-unstained necrotic tissue. The slices were subsequently fixed in 10 % formalin solution. The area at risk was determined by negative staining with Evan's blue. The areas of infarcted tissue, the risk zone, and the whole left ventricle (LV) were measured by computer morphometry using a Bioquant imaging software (BIO98). Infarct size was expressed both as a percentage of the ischemic risk area.

Measurement of hemodynamics

Hemodynamic measurements included heart rate and mean arterial pressure, systolic and diastolic blood pressure. Rate-pressure product was calculated as the product of heart rate and peak arterial pressure.

Study Protocol

All animals were subjected to infarction protocol consisting of 30 minutes of sustained ischemia by occlusion of coronary artery followed by 180 minutes of reperfusion. The effect of sildenafil was studied in the absence or presence of 5-HD in two phases i.e., the acute and delayed phase. In the acute phase, myocardial infarction protocol was carried out 30 min after treatment with sildenafil. In the delayed phase, ischemia/reperfusion protocol was carried out 24 hours later. The rabbits were randomly assigned into one of the following groups. Following is the description of the experimental groups:

Group I: (saline control, n=10): Rabbits received 0.9 % saline; **Group II: (Sildenafil, acute phase, n=6):** The Viagra tablets were crushed and 0.7 mg/kg sildenafil was dissolved in 3 ml saline. This preparation was given as IV bolus, approximating on a

mg/kg basis, the clinical dose of 50 mg administered to a 70 kg patient as described by Przyklenk and Kloner (15). The animals were subjected to ischemia/reperfusion 30 minutes later. **Group III: (Sildenafil, delayed phase, n=6).** Animals were treated with sildenafil as in group II and subjected to ischemia/reperfusion 24 hrs later; **Group IV: (5HD, n=7).** Control rabbits treated with 5-HD (5 mg/kg, IV) 10 min prior to sustained ischemia and reperfusion; **Group V: (Sildenafil, + 5HD, acute phase, n=6).** Sildenafil treated rabbits as in group II were given 5-HD (5 mg/kg, IV) 10 min prior to ischemia and reperfusion; **Group VI: (Sildenafil, + 5HD, delayed phase, n=6).** Sildenafil treated rabbits as in group III were given 5-HD (5 mg/kg, IV) 10 min prior to ischemia and reperfusion.

In addition, a subset of three groups of animals (n=5-6 per group) were given sildenafil citrate orally (1.4 mg/kg) or saline (control) in order to determine the early and delayed cardioprotective effect of the drug through this route. Since there is 40 % bioavailability of sildenafil citrate after oral administration (http://www.pfizer.com/hml/pi's/viagrapi.pdf), we used double the dose of intravenous route i.e., 1.4 mg/kg which is equivalent to clinical dose of 100 mg for a 70 kg patient.

Statistics

All measurements of infarct size and risk areas are expressed as group means ± SEM. Changes in hemodynamics and infarct size variables were analyzed by a two-way repeated

measure ANOVA to determine the main effect of time, group and time by group interaction. If the global tests showed major interactions, post hoc contrasts between different time-points within the same group or between different groups were performed using t- test. Statistical differences were considered significant if p value was less than 0.05.

RESULTS

Hemodynamics: Intravenous administration of sildenafil citrate (0.7 mg/kg) caused rapid decrease in hemodynamcs as indicated by 24.5 %, 47.3 % and 38.8 % decline in systolic, diastolic and mean arterial pressure respectively within 2 minutes (Figure 1A). The systemic hemodynamics returned to nearly baseline levels by 5 minutes after treatment with sildenafil. No significant changes in heart rate were observed following treatment with sildenafil (not shown). The effect of orally administered sildenafil citrate on systemic hemodynamics was milder and slower as compared to the intravenous dose of the drug. The orally administered sildenafil caused approximately 9.2 %, 12.5 % and 10.3 % decrease in systolic, diastolic and mean arterial pressure respectively after 30 minutes of treatment with the drug (Figure 1B). This hypotensive response remained significant even at 60 minutes after oral administration of drug. No changes in heart rate were observed. Also, no significant changes in systemic hemodynamics were observed in control animals given saline orally (data not shown).

The heart rate, mean arterial pressure and rate pressure product during baseline, preischemia, 30 min of ischemia and 180 min of reperfusion period are shown in Table 1 and 2. The hemodynamics remained reasonably stable, although they gradually decreased in all the groups during experimental protocol. Except at the indicated time points, the mean values were not significantly different between the groups at any time point.

Infarct size

The infarct size (% of risk area) reduced from 33.8 ± 1.7 in the saline treated control group to 10.8 ± 0.9 during acute phase (68 % reduction, mean±SE, p< 0.05) and 19.9 ± 2.0 during delayed phase (41 % reduction) in the sildenafil-treated rabbits (Figure 2A). A similar reduction in infarct size was observed acutely (after 60 min) and 24 hrs later when sildenafil was administered orally (Figure B). The infarct-limiting effect of sildenafil was abolished in animals treated with 5-HD as shown by significant increase in infarct size to 35.6 ± 0.4 during acute phase and 36.8 ± 1.6 in the delayed phase (p<0.05 versus groups II and III treated with sildenafil, Figure 2A). Control animals treated with 5-HD had an infarct size of 33.5 ± 1.9 which was not different from infarct size of 33.8 ± 1.7 in the saline controls (P>0.05). A similar trend in the changes in infarct size was observed when expressed as a percentage of the left ventricle (not shown). Similarly, the risk areas expressed as percentage of left ventricle were not statistically significant different between

the groups. These data suggest that changes in the infarct size observed among various groups were not related to the percentage of the area of the left ventricle that was occluded by our technique. Representative sections of the heart treated with sildenafil citrate intravenously clearly demonstrated significantly larger area of viable tissue (brick red color) as compared to saline treated control and sildenafil+5-HD treated animals, which had much larger gray and white areas in the risk zone (Figure 3).

DISCUSSION

Sildenafil citrate (Viagra) is currently the only approved oral drug for treatment of erectile dysfunction in men. However, little is known about other beneficial effects of this drug. We report here our novel observation about the preconditioning-like effect of sildenafil in the adult rabbit heart. Our results show that intravenous administration of sildenafil induces acute (early) and delayed cardioprotective effect as indicated by significant reduction in the infarct size when compared to the saline-treated controls. Since the drug is taken orally by patients, we further showed that feeding the rabbits with sildenafil citrate reduced infarct size acutely (after 1 hr) as well as 24 hrs later which was comparable to the infarct size reduction obtained by intraveous administration of the drug. The selective blocker of mitoK$_{ATP}$ channels, 5-HD, when administered before the ischemia/reperfusion protocol, abolished both the early as well as delayed cardioprotection induced by sildenafil citrate. Intravenous administration of sildenafil citrate caused severe transient decrease in

the systemic hemodynamics (diastolic, systolic and mean arterial blood pressure) within 2 minutes after treatment returned to nearly baseline levels after 3 minutes. Although a significant decrease in systemic hemodynamics was also observed after oral administration of the drug, these changes were mild and occurred slowly. The hemodynamics remained largely unchanged among the groups during ischemia/reperfusion protocol.   To our knowledge, this is the first study demonstrating (a) the direct cardioprotective effect of sildenafil *in vivo* and (b) the involvement of mitoK$_{ATP}$ channel in mediating this protection in the ischemic heart.

Sildenafil is a potent selective inhibitor of PDE-5 in vascular smooth muscle cells which is known to enhance erectile function in men (20).   Sexual stimulation results in the release of NO from nerves and endothelial cells in the corpus cavernosum of the penis that stimulates guanylate cyclase with subsequent formation of cGMP.   Accumulation of cGMP leads to smooth muscle cell relaxation in the arteries, arterioles and sinusoids in the corpus cavernosum that allow this erectile tissue to fill with blood and causing erection (10). Men with erectile dysfunction may be unable to produce adequate amounts of cGMP because it may be broken down by PDE-5, which is found in high levels in the genitalia. Sildenafil inhibits PDE-5 allowing an increase in cGMP and improving vasodilation. Besides genitalia, PDE-5 is found in other vascular and visceral smooth muscles also (21). As a result, the administration of sildenafil causes vasodilation and decrease in the blood pressure. We hypothesized that such a vasodilatory action of sildenafil could potentially release endogenous mediators of preconditioning such as adenosine, bradykinin or NO.

One or more of these mediators may trigger signaling cascade leading to opening of mitoK$_{ATP}$ channel resulting in acute and delayed cardioprotective effect. Indeed our results show a very impressive acute cardioprotective effect which is comparable or even better than ischemic preconditioning (28) and pharmacological preconditioning induced by activation of adenosine receptors, monophosphoryl lipid A or bradykinin (9,13,17,26,27). The cardioprotective effect after 24 hrs was less pronounced during delayed phase i.e., 41% reduction in infarct size as compared to 67 % in the acute phase (% risk area), implying that there may be gradual waning of the sustained protective effect. Alternatively, it is possible that sildenafil-induced protection is biphasic, with acute and delayed phase protection controlled by separate mechanisms.

Another interesting observation in the present study is that both acute and delayed cardioprotective effects were blocked by 5-HD suggesting that opening of mitoK$_{ATP}$ channels play an important role in the infarct size reduction by sildenafil in our model. Several studies have now conclusively demonstrated that opening of mitoK$_{ATP}$ channels play an important role in ischemic as well as pharmacological preconditioning in the heart (7,8,18,22). Mitochondria are known to play an essential role in cell survival by ATP synthesis and maintenance of Ca^{2+} homeostasis. Opening of the mitoK$_{ATP}$ channel partially compensates the membrane potential, which enables additional protons to be pumped out to form a H^+ electrochemical gradient for both ATP synthesis and Ca^{2+} transport (16). The acute protection induced by sildenafil may be mediated by opening of the mitoK$_{ATP}$ channel either directly or through a variety of signaling pathways such as activation of

protein kinase C and MAP kinases. The delayed phase could be through the signaling cascade leading to the synthesis of inducible nitric oxide synthase, generation of NO and opening of the mitoK$_{ATP}$ channels as described previously (19,23,24,29-31). Currently we do not have the evidence in support of this notion and further investigations are needed to determine the cellular and molecular mechanisms of cardioprotection by sildenafil.

In the present studies, the sildenafil was given in normal rabbits with no sexual stimulation. It has been suggested that sexual activity is comparable to moderate exercise, particularly in men with coronary artery disease who may have been physically inactive to engage in sexual activity (10). Since exercise triggers preconditioning-like effect in animal models (25), it is possible that men taking sildenafil prior to sexual intercourse could potentially have additive cardioprotectve effect of moderate exercise. On the other hand, concerns over the safe use of sildenafil in patients with ischemic heart disease have been raised. While the overall safety of sildenafil use has been well established, the co-administration of long and short-acting nitrate preparations has been associated with significant hypotension and adverse cardiovascular effects. In addition, caution has been recommended in prescribing sildenafil to patients with unstable ischemic coronary syndromes, severe LV dysfunction, or patients who are on multiple-drug antihypertensive regimens (5, 12). Therefore, careful clinical studies are required to evaluate the preconditioning-like effect of sildenafil in patients with ischemic heart disease.

In conclusion, for the first time, we have demonstrated that intravenous or oral admininstration of sildenafil citrate induces significant cardioprotective effect against ischemia/reperfusion injury, the impact of which was powerful within 30 minutes and persisted to slightly lesser degree 24 hrs after administration of the drug. The extent of protection observed with sildenafil was comparable to preconditioning induced by sublethal ischemia and several other pharmacological agents. Furthermore, our results show that the cardioprotective effect of sildenafil was mediated by opening of $mitoK_{ATP}$ channel, a proposed end-effector of myocardial preconditioning (8,22). Further investigations are needed to understand the molecular mechanism(s) of sildenafil-induced cardioprotective effect which would help in expanding the utility of this drug for other cardiovascular diseases in addition to the current use for treatment of erectile dysfunction in men.

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Figure Legends

Figure 1: **Changes in hemodynamics following administration of sildenafil intravenously (A) or orally (B) administration of sildenafil citrate.** SBP – systolic blood pressure, DBP – diastolic blood pressure; MAP – mean arterial blood pressure. Results are means±SE from 6-7 rabbits.

Figure 2: A. Bar diagram showing infarct size (% risk area) after intravenous administration of sildenafil citrate. Saline control - Animals receiving 0.9 % saline; Sildenafil (acute phase) - Rabbits receiving sildenafil (0.7 mg/kg, IV) 30 min prior to ischemia/reperfusion; Sildenafil (delayed phase): animals receiving sildenafil (0.7 mg/kg, IV) 24 hrs prior to ischemia/reperfusion. **5-HD –** Control (saline-treated) rabbits received 5-HD (5 mg/kg) 10 min prior to sustained ischemia and reperfusion. **Sildenafil+5-HD** (*acute phase*): Sildenafil treated rabbits given 5-HD (5 mg/kg, IV) 10 minutes prior to sustained ischemia and reperfusion. **Sildenafil+5-HD** (*delayed phase*): Rabbits treated with sildenafil 24 hrs prior to ischemia/reperfusion were given 5-HD. Results are means±SEM in 6-7 rabbits in each group. *P < 0.05 compared to control, sildenafil, sildenafil + 5-HD (acute and delayed), and 5-HD groups.

B. **Reduction of infarct size (% of risk area) after oral administration of sildenafil citrate.** Rabbits were given sildenafil (1.4 mg/kg) or equivalent volume of saline prior to ischemia/reperfusion protocol which was carried out after 60 min (acute phase) and 24 hrs later (for delayed phase).

Figure 3: **Representative sections of the heart demonstrating reduction of post-ischemic infarct size 30 minutes following treatment with sildenafil and blockade of protective effect with 5-HD.** At the end of experimental protocol as described in Methods, the hearts were perfused with Evan's blue to demarcate the risk area. Each heart was then sliced into 4-5 sections and stained with TTC followed by fixation in formalin. The blue areas represent normal perfused tissue. The viable areas are stained brick red whereas infracted are gray or white. Note that significant viable area in the sections of the heart treated with sildenafil as compared to saline control or salidenafil + 5-HD treated rabbit. Similar pattern was observed in the sections of the heart from delayed phase groups (not shown).

Figure 1

Figure 1.

A.

B.

Figure 2

Figure 2

(A)

(B)

Figure 3

Saline (Control)

Sildenafil (0.7 mg/kg)

Sildenafil (0.7 mg/kg) + 5-HD
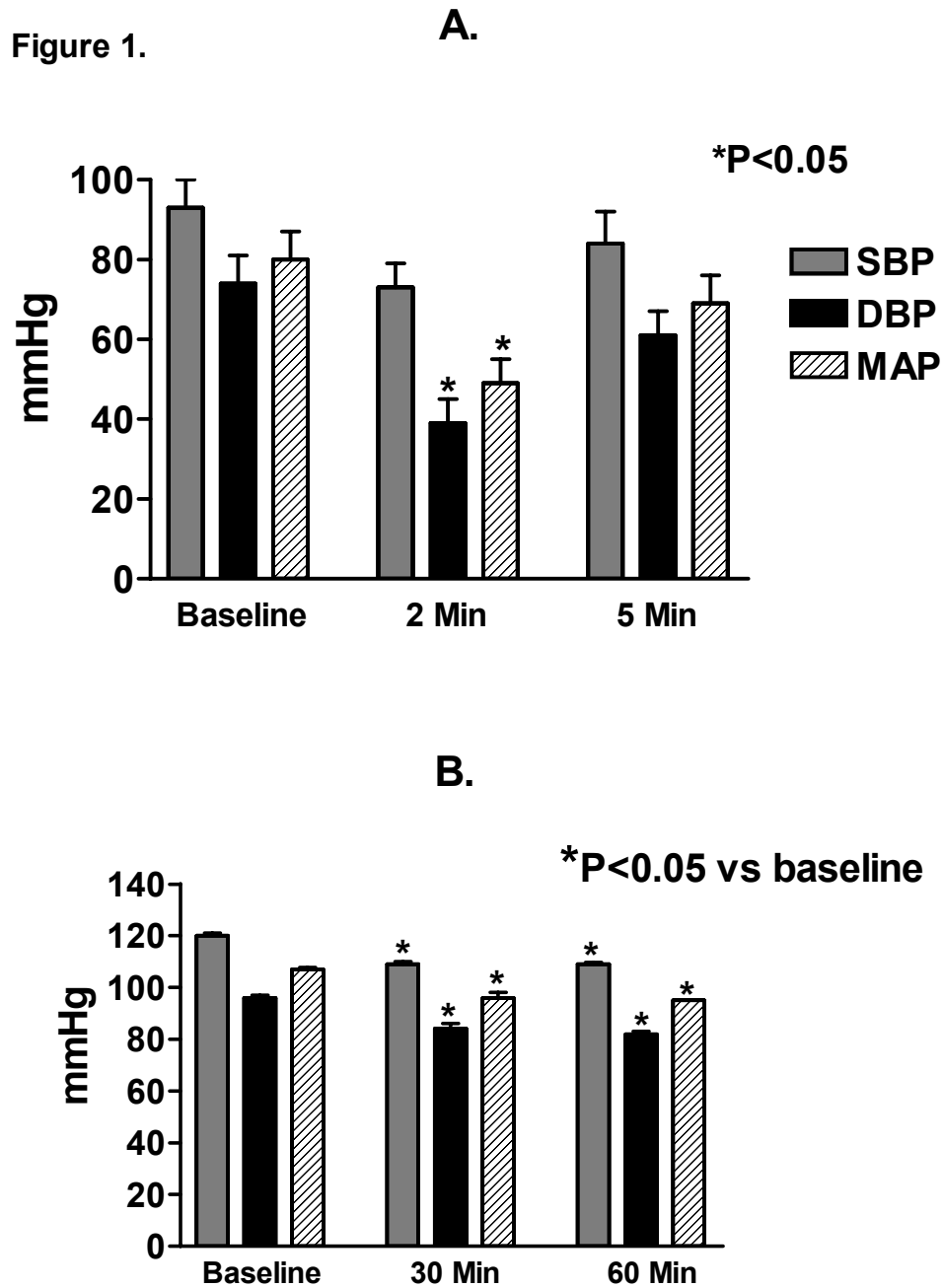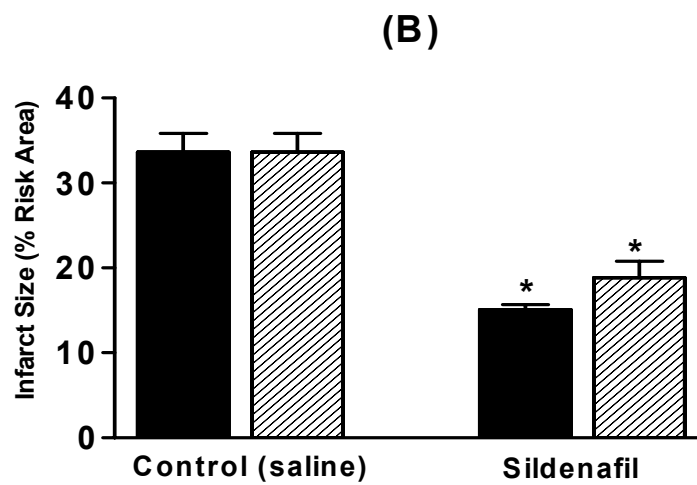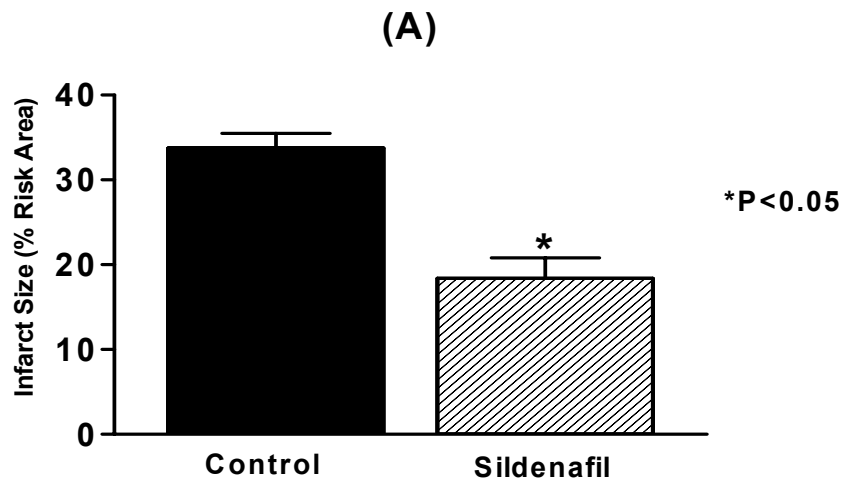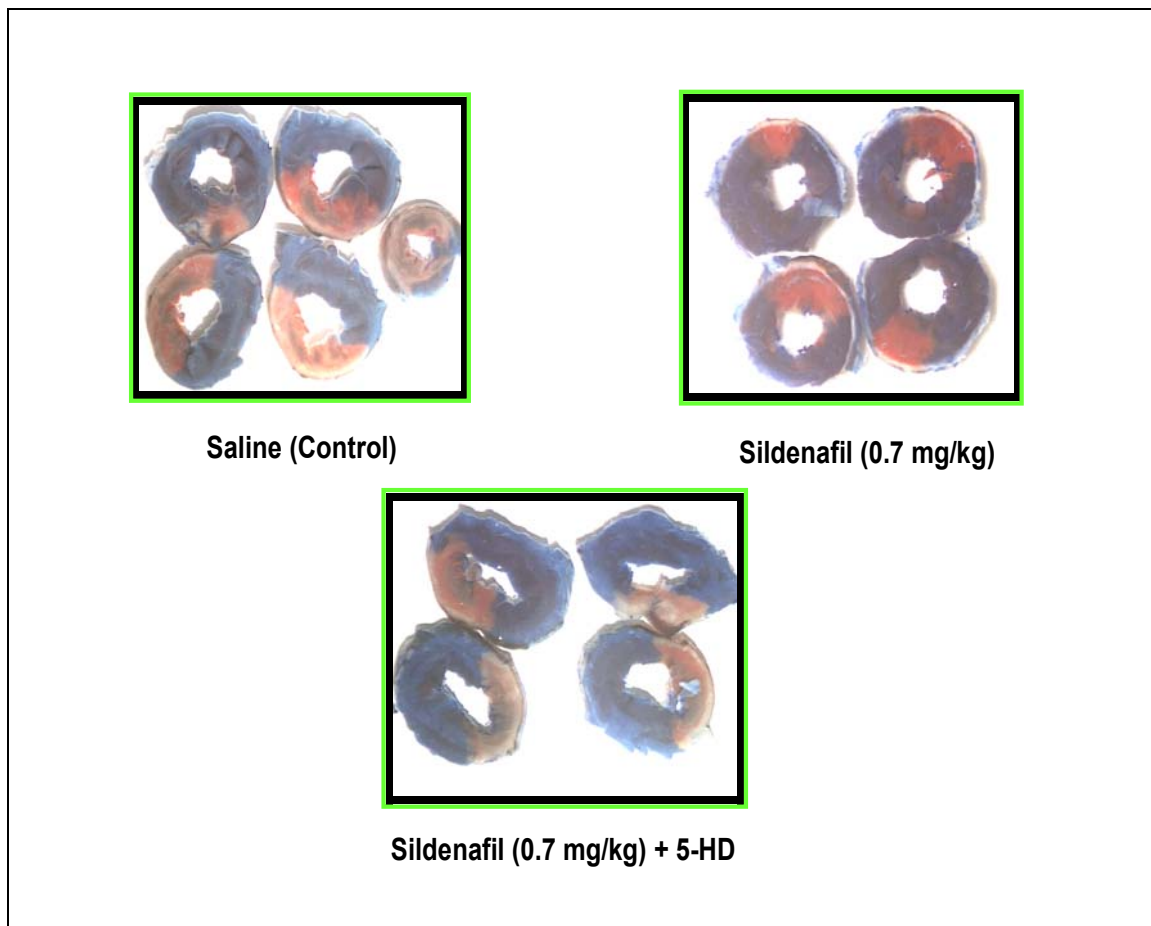
CHAPTER 3

Sildenafil Induces Delayed Preconditioning Through iNOS-Dependent Pathway in Mouse Heart

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Abstract

Sildenafil citrate (Viagra) is the most widely used drug for treating erectile dysfunction in men. We recently demonstrated that it induces potent protective effects against ischemia-reperfusion (I-R) injury in rabbit hearts through opening of mitochondrial K_{ATP} channels. In the present study, we investigated the role of nitric oxide (NO)-dependent signaling pathway in delayed cardioprotection by sildenafil. Adult male ICR mice were treated with Saline or Sildenafil (0.7 mg/kg, *i.p.*) 24 hours before global I-R in

Langendorff mode. Infarct size was reduced from 27.6±3.3% in saline controls to 6.9±1.2% in sildenafil-treated mice (means±SEM, $P<0.05$) without compromising cardiac function. RT-PCR revealed a transient increase in endothelial and inducible NO synthases (eNOS and iNOS) mRNA in sildenafil-treated mice, peaking at 45 min (eNOS) and 2 hours (iNOS) after sildenafil injection. The magnitude of mRNA increase was more pronounced for iNOS than eNOS. In addition, a significant increase in both iNOS and eNOS protein was detected 24 hours after sildenafil treatment. Selective inhibitor of iNOS, 1400W (10 mg/kg, *i.p.* given 30 min before I-R) abolished sildenafil-induced protection (23.7±2.8%, $P<0.05$ *vs.* Sildenafil). These data suggest that induction of NOS isoforms is an essential component of signaling mechanism for sildenafil-induced delayed preconditioning. However, iNOS appears to be the primary isoform that mediates the robust cardioprotection.

Introduction

Sildenafil citrate (Viagra) is a selective inhibitor of phosphodiesterase-5 (PDE-5) that catalyzes the breakdown of cGMP – one of the primary factors involved in smooth muscle relaxation. It enhances nitric oxide (NO)-driven cGMP accumulation which in turn causes vasodilatation in *corpus cavernosum*. Sildenafil has become the most widely used drug for treating erectile dysfunction (ED) in men since its market debut in 1998.[1] Interestingly, we recently discovered a powerful preconditioning-like effect of sildenafil in rabbit hearts.[2] Both intravenous and oral administration of sildenafil caused significant reduction of

infarct size following ischemia/reperfusion. The protection was abolished by 5-hydroxydecanoate, a selective blocker of mitoK$_{ATP}$ channels.[2] However, the mechanism by which sildenafil triggers signaling cascade leading to opening of mitoK$_{ATP}$ remains speculative[2]. There is mounting evidence suggesting a role of NO in modulating mitoK$_{ATP}$.[3-6] The synthesis of NO is catalyzed by three isoforms of NO synthase, namely, neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), among which iNOS has been identified as the essential mediator of delayed preconditioning induced by divergent pathophysiological stimuli or pharmacological agents, such as brief episodes of ischemia-reperfusion (I-R),[7] endotoxin derivatives,[8,9] G protein-coupled membrane receptor agonists,[10,11] whole body hyperthermia,[12] and systemic hypoxia.[13] However, no studies are available showing any link between sildenafil and activation of NOS-dependent signaling cascade. The goal of the present report was to show if: 1) sildenafil induces synthesis of NOS isoforms in the heart and, 2) iNOS mediates the delayed preconditioning effect in the mouse heart.

Materials and Methods

Physiological Studies. Adult male out-bred ICR mice were supplied by Harlan. Animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Viagra pills (Pfizer Inc.) were ground into powder and dissolved in saline. The drug solution was filtered (0.45 μm pore size) before intraperitoneal (*i.p*) injection. iNOS inhibitor – 1400W was obtained from Alexis.

We used Langendorff isolated perfused mouse heart model subjected to 30 min of global ischemia and 30 min of reperfusion. Myocardial I-R injury was assessed by measuring infarct size, contractile function, and coronary flow as described previously.[9]

Twenty eight mice were randomized into the following four groups (n = 6-9/group): 1) *Saline* (0.15 ml, *i.p.*); 2) *Sildenafil* (0.7 mg/kg, *i.p.*, equivalent to 50 mg Viagra pill used for ED patients, given 24 hrs befre I-R); 3) *Sildenafil+1400W* (10 mg/kg, *i.p.*,[14] given 30 min prior to I-R); and 4) *Saline+1400W*.

Measurement of NOS isoforms. Mice were treated with sildenafil (0.7 mg/kg, *i.p.*), hearts were removed at 15 min, 30 min, 45 min and 1, 2, 3, 4, 24 hrs post injection (n = 3/group). Three non-treated hearts were used as controls. Tissue samples were ground under liquid nitrogen and homogenized with TRI Reagent (Molecular Research Center) for extracting total RNA, which was reverse-transcribed into cDNA at 50 $^{\circ}$C for 30 min using OneStep RT-PCR Kit from Qiagen. The oligonucleotide primers were synthesized based on published sequences for murine iNOS, eNOS, and GAPDH.[15] (Integrated DNA Technology). The RT-PCR products were electrophoresed on 1.5% Tris-Acetate-EDTA agarose gel. The target bands were identified based on their specific size using DNA standards.

iNOS and eNOS proteins were measured by Western blots as described previously.[13] In brief, triplicate heart samples were collected 24 hours after saline or sildenafil injection and homogenized in ice-cold RIPA buffer (Upstate Biotechnology). The homogenate was centrifuged at 10,000g for 10 min under 4°C, and supernatant was recovered as the total

cellular protein. 60 μg total protein from each sample was separated by SDS/PAGE on 10% acrylamide gels and transferred to a PVDF membrane, and then blocked with 5% non-fat dry milk in TBS. The membrane was subsequently incubated with a rabbit polyclonal antibody (Santa Cruz; dilution 1:500) reacting specifically to iNOS, eNOS, or actin. The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, 1:1,000 dilution). The membranes were developed using enhanced chemiluminescence and exposed to x-ray film. The mRNA and protein expression were quantified by scanning each of the RT-PCR or Western blot band using a densitometer (Bioquant 98).

Data Analysis and Statistics. Data are presented as means±SEM. The difference among the treatment groups or the time points after sildenafil injection was compared by unpaired *t* test or one-way ANOVA followed by Student-Newman-Keuls post-hoc test. $P<0.05$ was considered significant.

Results and Discussion

Pretreatment of mice with sildenafil reduced infarct size 24 hrs later (6.9±1.2%), as compared to saline-treated controls (27.6±3.3%; $P<0.05$, Figure 1). The infarct-limiting effect of sildenafil was not associated with compromised ventricular contractile function, *i.e.* stunning (Figure 1, Bottom panel), which is in agreement with the results of Przyklenk and Kloner.[16] Sildenafil did not alter pre- or post-ischemic coronary flow (data not shown), indicating its cardioprotective effect may be independent of its vascular response 24 hrs later.

These results confirmed our previous findings showing a powerful cardioprotective effect of sildenafil in rabbit heart.[2]   There was increase in iNOS and eNOS mRNA and protein expression (Figure 2 and 3).   The levels of these transcripts increased transiently, peaking at 45 min (eNOS) and 2 hrs (iNOS) after sildenafil treatment and returning to baseline levels several hours later (Figure 2).   The magnitude of increase for iNOS was much higher compared with eNOS.   In addition, sildenafil-induced protection was abolished by selective iNOS inhibitor, 1400W (Infarct size, $23.7 \pm 2.8\%$, $P<0.05$ *vs. Sildenafil*).   1400W had no significant effect on infarct size as compared with saline-treated hearts ($24.5 \pm 1.0\%$; $P>0.05$ *vs. Saline*).

Several studies have shown that NO derived from iNOS plays a major role in delayed cardioprotection induced by endotoxin derivatives,[8,9] agonists of adenosine or adrenergic receptors,[10,11] p38 activator,[17] and mitoK$_{ATP}$ opener – diazoxide.[6]   The role of sildenafil in stimulating the release of NO in heart is unknown.   We provide the first evidence that sildenafil is a potent inducer of iNOS mRNA and protein, which leads to delayed cardioprotection.   Although eNOS mRNA and protein also increased, their quantitative expression was lower than iNOS.   The role of eNOS is not clear in the present study.   The complete blockade of cardioprotection with 1400W given before ischemia rules out the role of eNOS in the mediator phase of delayed protection.   However, eNOS may play a role in the trigger phase, *i.e.* at the time of sildenafil treatment when NO from eNOS may initiate the signaling cascade leading to iNOS expression as proposed by Bolli and coworkers.[18,19]   The iNOS-catalyzed NO generation could potentially activate guanylate cyclase resulting in enhanced formation of cGMP.   cGMP may activate protein kinase G

(PKG) that can subsequently open mitochondrial K_{ATP} channel resulting in the cardioprotective effects as recently reported.[20]

To conclude, for the first time, our results show that sildenafil induces delayed preconditioning which is primarily mediated by NO derived from iNOS. Further studies are needed to understand the signaling mechanism(s) that lead to transcription and expression of eNOS and iNOS in the heart. The current study in a model of global I-R further expands our knowledge on the cardioprotective effect of sildenafil, which may potentially be used for treatment of patients with ischemic heart diseases.

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Legend of Figures

Figure 1. Effect of sildenafil and/or 1400W on myocardial infarct size (*Top*) and ventricular functional recovery (*Bottom*) following global ischemia-reperfusion.

Figure 2. Time course of iNOS and eNOS mRNA expression determined with RT-PCR following sildenafil treatment. Top panel shows representative pictures of 3 independent experiments. The scatter plots present the densitometric results averaged from 3 individual hearts for each time point, which were normalized against GAPDH level for each sample.

Figure 3. Cardiac expression of iNOS and eNOS protein 24 hrs after sildenafil treatment. The bar graph shows the densitometric quantification averaged from 3 individual hearts for each group, which is normalized against Actin level for each sample.

Figure 1

Infarct Size

Legend:
- Saline (n=6)
- Sildenafil (n=9)
- Sildenafil+1400W (n=7)
- Saline+1400W (n=6)

* $P<0.05$

Cardiac Function

Figure 2

Figure 3

iNOS

eNOS

Actin

Saline   Sildenafil

(24 hours after treatment)
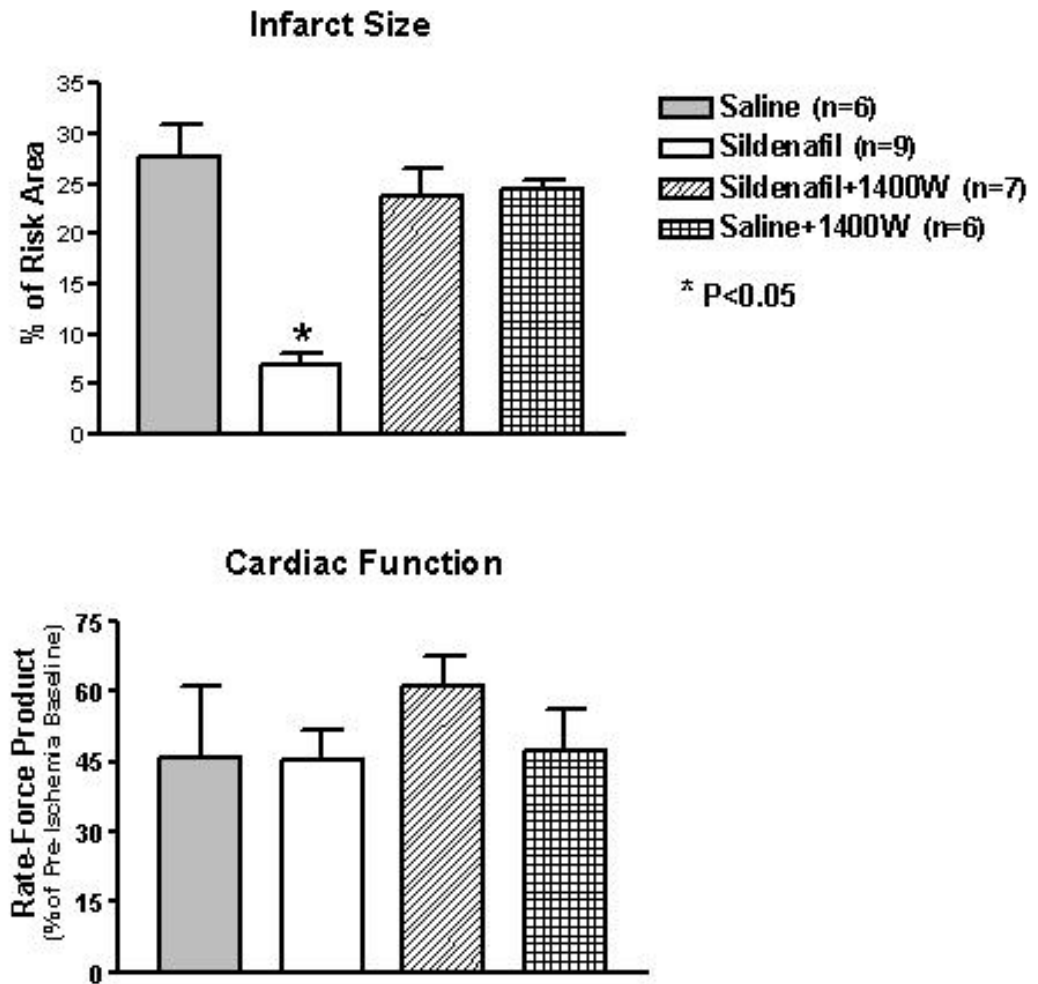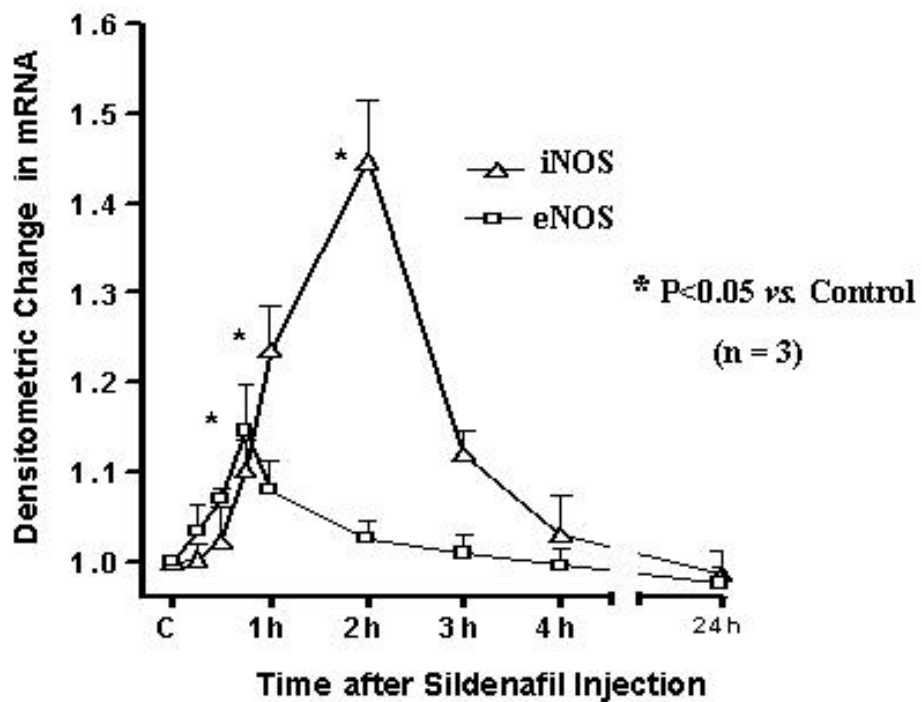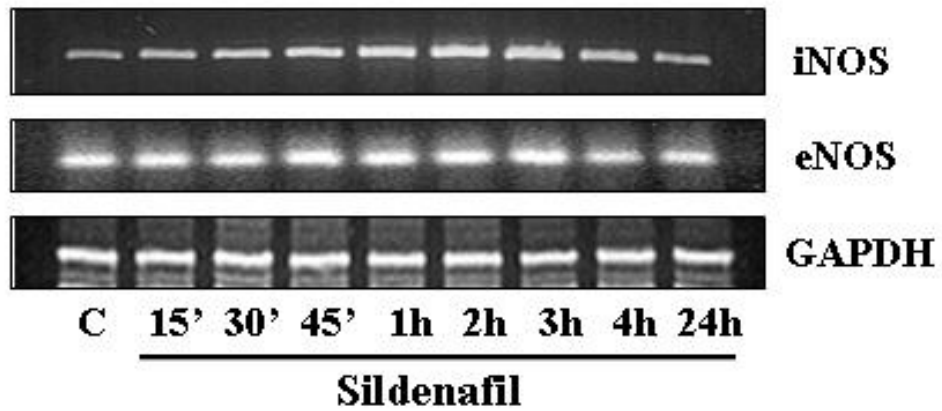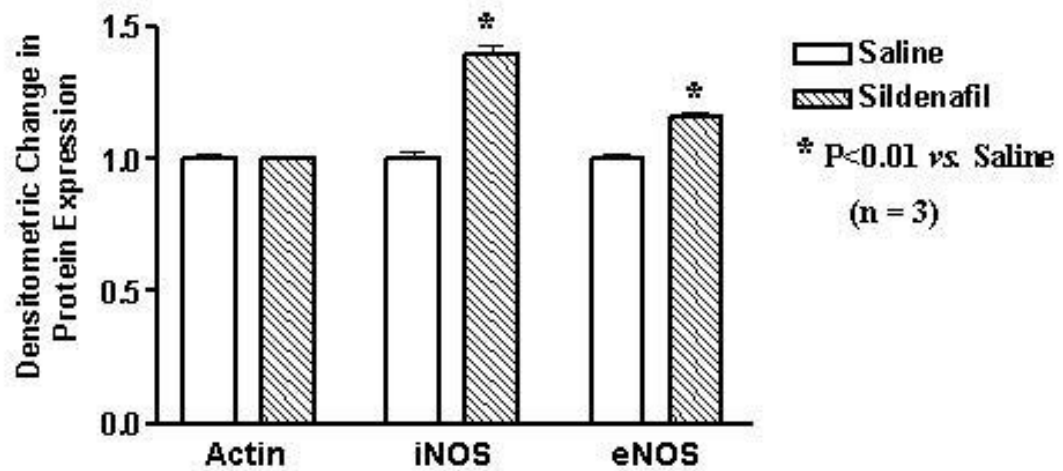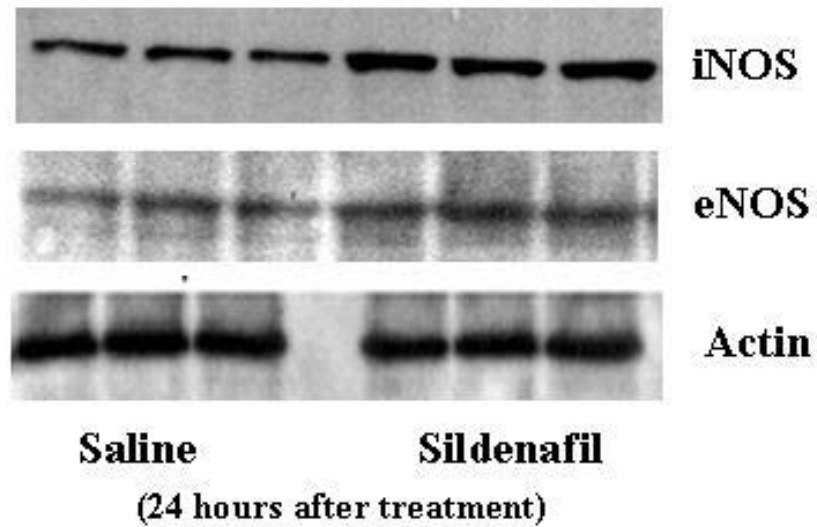
CHAPTER 4

Protein kinase C plays an essential role in sildenafil-induced cardioprotection in rabbits

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ABSTRACT

Sildenafil citrate (Viagra) is the most widely used pharmacological drug for treating erectile dysfunction in men. It has potent cardioprotective effects against ischemia-reperfusion (I/R) injury via nitric oxide and opening of mitoK$_{ATP}$ channels. We further investigated the role of protein kinase C (PKC)-dependent signaling pathway in sildenafil-induced cardioprotection. Rabbits were treated (orally) with sildenafil citrate (1.4 mg/kg, iv) 30 min prior to index ischemia for 30 min and reperfusion for 3 hrs. PKC inhibitor, chelerythrine (5 mg/kg, iv) was given 5 min prior to sildenafil. Infarct size (% of risk area) reduced from 33.65 ±2.17 in vehicle (saline) group to 15.07±0.63 in sildenafil-treated groups, a 45 % reduction compared to the vehicle (mean ± SEM, p<0.05). Chelerythrine abolished sildenafil-induced protection as demonstrated by increase in infarct size to

31.14±2.4 (p<0.05). Chelerythrine alone had an infarct size of 33.5±2.5, which was not significantly different when compared to DMSO treated group (36.8±1.7, P>0.05). Western blot analysis demonstrated translocation of PKC-α, -θ and -δ isoforms from cytosol to membrane after treatment with sildenafil. However, no change in PKC-β and -ϵ isoforms was observed. These data provide direct evidence of an essential role of PKC, and potentially PKC-α, -θ and -δ in sildenafil-induced cardioprotection in rabbit heart.

Introduction

Sildenafil Citrate (Viagra), a specific phosphodiesterase-5 (PDE-5) inhibitor currently approved for the treatment of erectile dysfunction in men, has been shown to acutely enhance endothelium-dependent vasodilation in patients with heart failure (7). This drug allows accumulation of the potent relaxing agent cGMP in vascular smooth muscle cells by preventing its breakdown by PDE-5. Sildenafil has been shown to enhance nitric oxide (NO)-driven cGMP accumulation in the corpus cavernosum of rabbits without affecting cAMP formation (33). It causes mild to moderate decrease in systolic and diastolic pressure because of the inhibition of PDE-5 in smooth muscle cells in the vascular bed (13). Interestingly, our laboratory recently observed that sildenafil induced both acute and delayed cardioprotective effects against ischemia-reperfusion injury in rabbits which was dependent on the opening of mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel (23). In addition, we demonstrated that sildenafil induced delayed preconditioning in the mouse heart through increased expression of inducible nitric oxide

synthase (iNOS) (31). However, the signaling mechanism involved in cardioprotection is currently unknown.

Several studies suggest that protein kinase C (PKC) activation plays an important role in the mechanism of preconditioning (1, 2, 15, 17, 26, 37). The PKC family of isozymes has three major subgroups: the conventional, calcium-dependent (α-, ßI-, ßII-, and γ), the novel, and calcium-independent PKC isozymes (δ-, ε-, η-, θ-, and possibly μ). Translocation of PKC isoform(s) from the cytosolic to the particulate fraction (including sarcolemmal, mitochondrial as well as nuclear fractions) result in their binding to specific receptors of activated C kinase (RACKs) localized in membranes (20, 21). Translocated specific PKC isoforms are believed to participate in several functions including the opening of mitoK$_{ATP}$ channels or the induction of gene expression (9, 34, 35). It has been shown that PKC-mediated cardioprotection is isoform specific: the ε and η-isoforms play an essential role in the development of ischemic preconditioning in rabbit myocardium (4, 15, 16, 27, 29). In addition, a number of pharmacological agents cause cardioprotective effect through selective translocation of PKC isoforms to the membrane fractions (14, 32, 40). However, the role of PKC or translocation of specific isoform(s) following sildenafil induced cardioprotection is not known. Accordingly, the goal of the present investigation was (a) to show if the cardioprotective effects of sildenafil is blocked by the PKC inhibitor, chelerythrine in the rabbit heart and (b) to determine whether sildenafil induces translocation of specific isoform(s) of PKC from the cytosolic to the particulate fraction.

MATERIALS AND METHODS

Experimental Protocol. All animals were subjected to an infarction protocol consisting of 30 min of sustained ischemia by occlusion of the coronary artery followed by 180 min of reperfusion (Figure 1A). The effect of sildenafil was studied in the absence or presence of chelerythrine chloride. The myocardial infarction protocol was carried out 60 min after treatment with sildenafil. The rabbits were randomly assigned into one of the following groups. Group I: (saline control, n=6): Rabbits received 0.9 % saline; Group II: (Sildenafil, n=6): The Viagra tablets were crushed, dissolved in water and given orally to the rabbits. Because there is 40% bioavailability of sildenfil citrate after oral administration, we used double the dose of the intravenous route; i.e. 1.4 mg/ kg, which is equivalent to clinical dose of 100 mg for a 70-kg patient. Our previous study (23) has shown that an oral dose of 1.4 mg/kg is as potent as the intravenous dose of 0.7 mg/kg in cardioprotection. In addition, a group of animals were given sildenfil cytrate (0.7 mg/kg) as an IV bolus, the clinical dose of 50 mg administered to a 70 kg patient (28). Group III: (Sildenafil + Chelerythrine): Chelerythrine chloride was dissolved in DMSO (25 mg/2.5ml DMSO) and 5 mg/kg chelerythrine was administered 5 min prior to sildenafil treatment. Group IV: (Cheleryhtrine): Chelerythrine was given alone without sildenafil.

Group V (DMSO): DMSO, the solvent for chelerythrine, was administered 35 min prior to ischemia/reperfusion protocol.

Surgical Preparation. The surgical protocol is identical to that discussed in Chapter 2.

Measurement of Hemodynamics. The method of measuring hemodynamics used in Chapter 2 was applied in this study.

Measurement of subcellular distribution of PKC isozymes. For measurement of PKC, rabbits were administered sildenafil (1.4 mg/kg) or saline orally and hearts were excised 30, 60 and 120 minutes later (Figure 1B). The left ventricle was dissected and stored in liquid nitrogen until used. The frozen tissue samples were ground in a pre-chilled mortar and pestle under liquid nitrogen. Total cellular proteins were obtained by glass-glass homogenization of the powdered tissue in 3 ml extraction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 50µg/ml PMSF, protease inhibitor cocktail (10µl/ml, Sigma, product No. P8340), 0.3% β-mercaptoethanol as described by Qiu et al. (29). The homogenates were centrifuged at 45,000g for 30 min at 4°C. The supernatant containing cytosolic protein was saved, and the pellet was re-suspended in 3 ml of same extraction buffer along with 1% (v/v) Triton X-100 and incubated on ice for 1 hr and centrifuged at 45,000g for 30 min to obtain supernatant (particulate fraction). The protein concentration was determined using Bio-Rad Protein Assay kit (BioRad, Hercules, CA).

Cytosolic and membrane fractions (100 µg protein) were separated by SDS-PAGE on 10% denaturing acrylamide gels and transferred to nitrocellulose membranes. The blots were blocked with 5 % nonfat dry milk in TBST buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl and 0.1% Tween 20) for 1h. The blots were then incubated with 1,000 fold diluted primary antibodies against respective PKCs (Santa Cruz Biotechnology, California; cPKCα (SC-8393); cPKCβ$_1$, (SC-8049); nPKCδ (SC 8402); nPKCε (SC-1681); nPKCθ (SC-1680) to assess the expression of individual PKC isoform. To normalize for loading of protein, we used ß-actin antibody (Sigma, A-2172). After 2 hrs of incubation with respective primary antibodies, the blots were washed and incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2000 dilution, Amersham Pharmacia Biotech, UK) for 1hr. The blots were developed with the use of a chemiluminescent system (ECL kit, Amersham). Each immunoblotting experiment was repeated twice and the results were averaged. For quantifying the protein translocation, the optical density for each blot was scanned and analyzed with a densitometric system (Bioquant 98).

RESULTS

Infarct size. Figure 2A shows infarct size expressed as the percentage of risk area. Preconditioning with sildenafil resulted in a significant decrease in the infarct size from 33.65±2.17 in the control (saline) group to 15.07±0.63 in the sildenafil treated rabbits, a 45% reduction compared to the vehicle (mean ± SEM, p<0.05). The infarct size increased

significantly to 31.14±2.4, (p<0.05) with chelerythrine when given 5 minutes prior to sildenafil treatment. Chelerythrine alone had an infarct size of 33.5±2.5, which was not significantly different when compared to the DMSO (solvent of Chelerythrine) treated group (36.9±2.3). A similar trend in the changes in infarct size was observed when expressed as a percentage of the left ventricle (not shown). Similarly, the differences between the risk areas expressed as a percentage of the left ventricle were not statistically significant between the groups (Figure 2B). These data suggest that changes in the infarct size observed among various groups were not related to the percentage of the area of the left ventricle that was occluded by our technique. The infarct size (% of risk area) was reduced by 68% when sildenafil was administered intravenously (from 33.8 ± 1.7 in the saline-treated control group to 10.8 ± 0.9 in the sildenafil treated rabbits). This infarct size reduction <u>was</u> blocked by chelerythrine.

Hemodynamics. The heart rate, MAP and RPP are shown in Table 1. The heart rate, MAP and RPP remained reasonably stable throughout the experimental period, although they gradually decreased in most of the groups. Except at the indicated time points, the mean values were not significantly different between the groups at any time point within the groups.

Translocation of PKC Isoforms. We examined the sub-cellular distribution of five PKC isoforms (α, β_1, δ, ϵ and θ) after 30, 60 and 120 min of oral administration of sildenafil by Western blot with the use of isoform-specific antibodies. As shown in Figure

3, PKC isoforms α, β_1, δ, θ and ε isoforms were expressed in both cytosolic and membrane fractions. The subcellular distribution of these isoforms was generally higher in the cytosol as compared to the membrane fraction. No significant change of the translocation of PKCα occurred from cytosol to membrane after 30 minutes of sildenafil treatment compared to control (saline treament) (Fig.3A). However, an increase in membrane PKCα was observed at 60 minutes, which reached significantly higher levels by 120 minutes of sildenafil administration. PKCθ and δ exhibited significant increase of the translocation in the membrane fraction after 30 min to 120 min of sildenafil admistration compared to control (Fig. 3B, C). Quantitative analysis showed increase of PKCα/β-actin ratio in the membrane fraction by sildenafil from 0.52 ± 0.06 (in control) to 0.70 ± 0.04 after 120 min ($p<0.05$) (Fig.3A). The ratio of PKCθ/β-actin in the membrane fraction increased from 0.62 ± 0.06 in saline control to 0.87 ± 0.08 ($p<0.05$) after 120 min of sildenafil treatment (Figure 3B). Similarly, PKCδ/β-actin ratio in the membrane fraction increased from 0.74 ± 0.06 (control) to 0.94 ± 0.03 (sildenafil) ($p<0.05$) by 120 min of sildenafil treatment (Fig. 3C). In contrast no significant increase of translocation of PKCβ (Fig. 3D) and ε (Fig 3E) to membrane occurred following sildenafil treatment.

DISCUSSION

The use of sildenafil for treatment of erectile dysfunction by many patients with cardiovascular disease has resulted in a tremendous interest in the cardiovascular properties of the drug (3). Recently we reported that administration of sildenfil induced cardioprotection as indicated by significant reduction in the infarct size when compared to the controls (23). In addition, we showed that sildenafil induced delayed cardioprotective effect in the mouse heart through upregulation of iNOS and eNOS (31). The hypothesis behind these studies was that vasodilatory action of sildenafil could potentially release endogenous mediators of preconditioning such as adenosine, bradykinin, or NO. One or more of these mediators may trigger signaling cascade leading to opening of the mitoK$_{ATP}$ channel resulting in acute and delayed cardioprotective effects (23). There is substantial experimental evidence that one of the major intracellular signal transduction pathways controlling cardiac protection by ischemic preconditioning involves activation of PKC (24, 36). The question posed in this study was whether PKC plays a pivotal role in sildenafil-induced cardioprotection and to identify the specific isoform(s) may be involved in the protective role of sildenafil. Our data show that translocation of PKC -θ and -δ started to increase after 30 min of sildenafil treatment, reached to significantly increased level after 120 min of sildenafil treatment, which coincided with the ischemia protocol performed 60 min after oral administration of sildenafil. Although statistically non-significant, there was well-defined trend towards increase in the translocations of PKC -α, -θ and -δ with respect

to control at 30 and 60 min after sildenafil treatment. Our data shows that administration of chelerythrine, the blocker of PKC prior to sildenafil abolished the infarct limiting effect of sildenafil following ischemia/reperfusion. The reduction in infarct size was not altered by vehicle (i.e., DMSO) confirming that the blockade of protection was indeed due to chelerythrine only. Furthermore, chelerythrine did not have significant effect on infarct size in the sham ischemic/reperfused rabbit hearts suggesting that PKC antagonist interceded the signal transduction cascade during sildenafil treatment only. These data strongly indicate that PKC activation plays an important role in the signaling mechanisms leading to sildenafil-dependent cardiac protection in the rabbit heart.

Endogenous PKC exists in various isoforms with specific tissue distribution and sensitivity. Translocation of PKC from cytosolic to particulate compartments is commonly used as an index of PKC activation which is not only limited to sarcolemmal membrane but also to cytoskeletal, mitochondrial and nuclear fractions. In the present study, we identified and quantified different isoforms of PKC from cytosol and membrane fractions. Our results show significant translocation of PKC-α after 60 minutes of treatment with sildenafil. PKC-α is the major calcium dependent PKC isoform located in the soluble fraction in resting neonatal cardiac myocytes and an increase in calcium concentration selectively translocates PKC-α to the particulate fraction (30). The activation of PKC-α is essential for the regulation of the Raf-Ras-Erk cascade by IGF1 (insulin-like growth factor 1) (25) or by hypertrophic signaling in adult rat cardiomyocytes (10). Our results also showed significant translocation of PKC θ and δ from 30 min to 120 min by sildenafil.

While the translocation of PKC-α, δ and ϵ have been studied in various models of ischemic and pharmacological preconditioning, the role of PKC-θ in cardioprotection remains unknown. PKC-θ is present in T lymphocytes as well as skeletal muscle, and its role in T cell signaling has been studied extensively (11). In the primary myotubes from neonatal mouse hind limb muscle, a rapid translocation of PKC-θ to the membrane in response to treatment with the cholinergic receptor agonists, carbachol has been demonstrated (12). It was suggested that recruitment of PKC-θ to the membranes of myotubes following carbachol treatment plays a role in modulation of the function of membrane proteins, including receptors. Carbachol and PMA also caused an increase in PKC-α levels in the cytosol followed by its increase in membrane fractions (12). Inagaki et al. (6) showed that JTV519, a novel drug that has protective effect against Ca^{2+} overload-induced myocardial injury, provided anti-ischemic effect via specific activation of PKC-δ in rat hearts. Miyawaki and Ashraf (18) demonstrated that high calcium preconditioning evoked the translocation of PKC-α and PKC -δ to the cell membrane. In addition, PKC-ϵ was translocated to the intercalated disk and suggesting that PKC-ϵ may modulate myocardial function through cell-to-cell interactions. Kawamura et al., 1998 (8) showed that ischemic preconditioning translocates PKC-α, -δ and -ϵ, but translocation of PKCα was transient in isolated rat heart. Under low Ca^{2+} perfusion, preconditioning suppressed translocation of PKC-α, and -ϵ, but not PKC-δ suggesting that the translocation of calcium-independent PKC-δ is essential for mediating ischemic preconditioning. The difference of the preconditioning procedures may also influence the importance of the PKC isoforms in

mediating the protective effect (8). Pathophysiological stimuli including heat shock and combination of heat shock and ischemic preconditioning also resulted in the translocation of PKC-α and -δ in young rats (5) further supporting the importance of these isoforms for the signaling cascade in cardioprotection. The downstream targets of PKC mediated cardioprotection involves multiple signaling pathways which include activation of MAP kinases e.g. ERK 1/2 and p38 kinases (19, 22, 26, 38, 41). Recently, we demonstrated an essential role of PKC-δ in the delayed cardioprotection triggered by stimulation of adenosine A_3 receptor subtype in the mouse (40). In these studies, the selective early translocation of PKC-δ in the membrane fraction initiated downstream signaling involving activation of transcription factor NF$\kappa\beta$, generation of NO and opening of the mitochondrial K_{ATP} channels (39), the possible mediators of delayed pharmacological preconditioning in the heart.

In conclusion, for the first time we have demonstrated that sildenafil-induced cardioprotection is dependent on activation of PKC. Our results also show that selective translocation of three PKC isoforms (i.e., α, δ, θ) from cytosol to membrane fractions suggesting their potential role in sildenafil-induced cardioprotection. Further investigations are needed to determine the cause and effect of each of the translocated PKC isozymes in sildenafil-induced cardioprotection and to understand the mechanism(s) by which translocated isozymes orchestrate downstream targets involved in attenuation of ischemic injury. These studies would help in expanding our knowledge on using this drug for protection of ischemic myocardium in humans.

Acknowledgements

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FIGURE LEGENDS

Figure 1. Experimental protocols showing **(A)** Infarct study after oral administration of sildenafil citrate and/or intravenous administration of PKC inhibitor-chelerythrine and **(B)** PKC measurement following oral administration of sildenafil.

Figure 2. Bar diagram illustrates infarct size (% risk area) **(A)** and risk area (% left ventricle) **(B)** after oral administration of sildenafil citrate and/or intravenous administration of PKC inhibitor- chelerythrine following ischemia and reperfusion. Saline control, rabbits received 0.9 % saline. Sil, rabbit received 1.4 mg/kg sildenafil 60 min before ischemia-reperfusion. Sil + Che, chelerythrine was administered 5 min. prior to sildenafil treatment. Che, chelerythrine was given alone 65 min before ischemia/reperfusion. DMSO, the solvent for chelerythrine was administered alone 65 min prior to ischemia/reperfusion protocol. Results are means ±SE in 6 rabbits in each group. *P< 0.05 compared with saline control, sildenafil, sildenafil + chelerythrine , chelerythrine, and DMSO.

Figure 3. Effect of sildenafil on subcellular localization of PKC-α **(A)**, -θ **(B)**, -δ **(C)**, -β **(D)** and -ϵ **(E)** in rabbit heart. Western blot showed subcellular distribution of PKC isoforms between cytosolic and membrane fractions after 30 min (S-30min), 60 min (S-60min) and 120 min (S-120min) treatment with sildenafil. Densitometric analysis showed

the average of the ratio of each isoform with respect to β-actin level from 4 individual hearts per treatment group. *P< 0.05 compared with saline control and S-120 min.

ABBREVIATIONS : Che, Chelerythrine; PKC, Protein kinase C; Sil,

Sildenafil Citrate(Viagra).

Table 1. Hemodynamic Data

	Baseline	Pre-ischemia	*30-min Ischemia*	*Reperfusion (60 min)*	*Reperfusion (120 min)*	*Reperf (180 min)*
Group I. (Saline Control, n=6)						
HR	199 ± 8	213 ± 9	209 ± 9	191 ± 10	182 ± 10	179 ± 10
MAP	94 ± 3	84 ± 4^d	$79 \pm 4d$	73 ± 3^d	$72 \pm 3d$	$67 \pm 2^{d,e}$
RPP	$21072 \pm 950^{a,b,c}$	20896 ± 842	19067 ± 781^a	$16138 \pm 855^{a,d,e,f}$	$15341 \pm 978^{d,e,f}$	$13969 \pm 819^{a,d,e}$
Group II. (Sildenafil, n=6)						
HR	216 ± 5	210 ± 20	216 ± 18	205 ± 14	187 ± 9	187 ± 9
MAP	100 ± 2	95 ± 3	96 ± 6	89 ± 0.4	82 ± 5^d	81 ± 5^d
RPP	24859 ± 239	22848 ± 2027	23137 ± 1617	21647 ± 2274	17738 ± 860^d	18555 ± 1336
Group III. (Sildenafil + Chelerythrine, n=6)						
HR	181 ± 18	188 ± 9	172 ± 15	194 ± 19	182 ± 17	186 ± 18
MAP	102 ± 6	93 ± 4	78 ± 5^d	69 ± 5^d	72 ± 5^d	67 ± 11^d
RPP	$14,573 \pm 1579^{a,e}$	$19,906 \pm 688$	$15,002 \pm 1181^{a,e}$	$14,644 \pm 964^{a,e}$	$14,647 \pm 1482^e$	$14,261 \pm 1330^{a,e}$
Group IV. (Chelerythrine, n=6)						
HR	210 ± 8	200 ± 7	188 ± 8	178 ± 7	171 ± 7^d	168 ± 11^d
MAP	88 ± 4	84 ± 2	80 ± 4	78 ± 6	70 ± 6	71 ± 6
RPP	$23,478 \pm 1021^{b,c}$	$20,259 \pm 1202$	$18,398 \pm 1475^{a,d}$	$14,901 \pm 975^{a,d,e}$	$16,987 \pm 1215^d$	$13,579 \pm 986^{a,d,e,f}$
Group V. (DMSO, n=6)						
HR	177 ± 11	200 ± 5	209 ± 4	200 ± 16	195 ± 4	194 ± 2
MAP	90 ± 16	77 ± 11	66 ± 4^a	66 ± 11	68 ± 12	67 ± 10
RPP	$17,585 \pm 846^{a,b}$	$18,345 \pm 1021$	$15,175 \pm 625^{a,e}$	$13,902 \pm 423^{a,d,e}$	$15,313 \pm 213^e$	$15,102 \pm 547^{a,e}$

Values are means±SEM. HR-Heart Rate (beats/min); MAP-Mean Arterial Blood Pressure (mmHg); RPP- Rate Pressure Product (mmHg) ^{a}P<0.05 vs.Sildenafil; ^{b}P<0.05 vs.Sildenafil+ Chelerythrine; ^{c}P<0.05 vs DMSO; ^{d}P<0.05 vs. Baseline; ^{e}P<0.05 vs. Pre-ischemia;^{f}P<0.05 vs. 30-min Ischemia.

Figure 1

A

B

Figure 2

*P<0.05
n=6

A

B

n=6

Figure 3

Figure 3

C

| Cytoplasmic | Membranous |

PKCδ →

PKCδ/β-Actin

PKCδ/β-Actin Ratio

Cytoplasmic   Membranous

D

| Cytoplasmic | Membranous |

PKCβ →

PKCβ/β-Actin

PKCβ/β-Actin Ratio

Cytoplasmic   Membranous

E

| Cytoplasmic | Membranous |

PKCε →

PKCε/β-Actin

PKCε/β-Actin Ratio

Cytoplasmic   Membranous
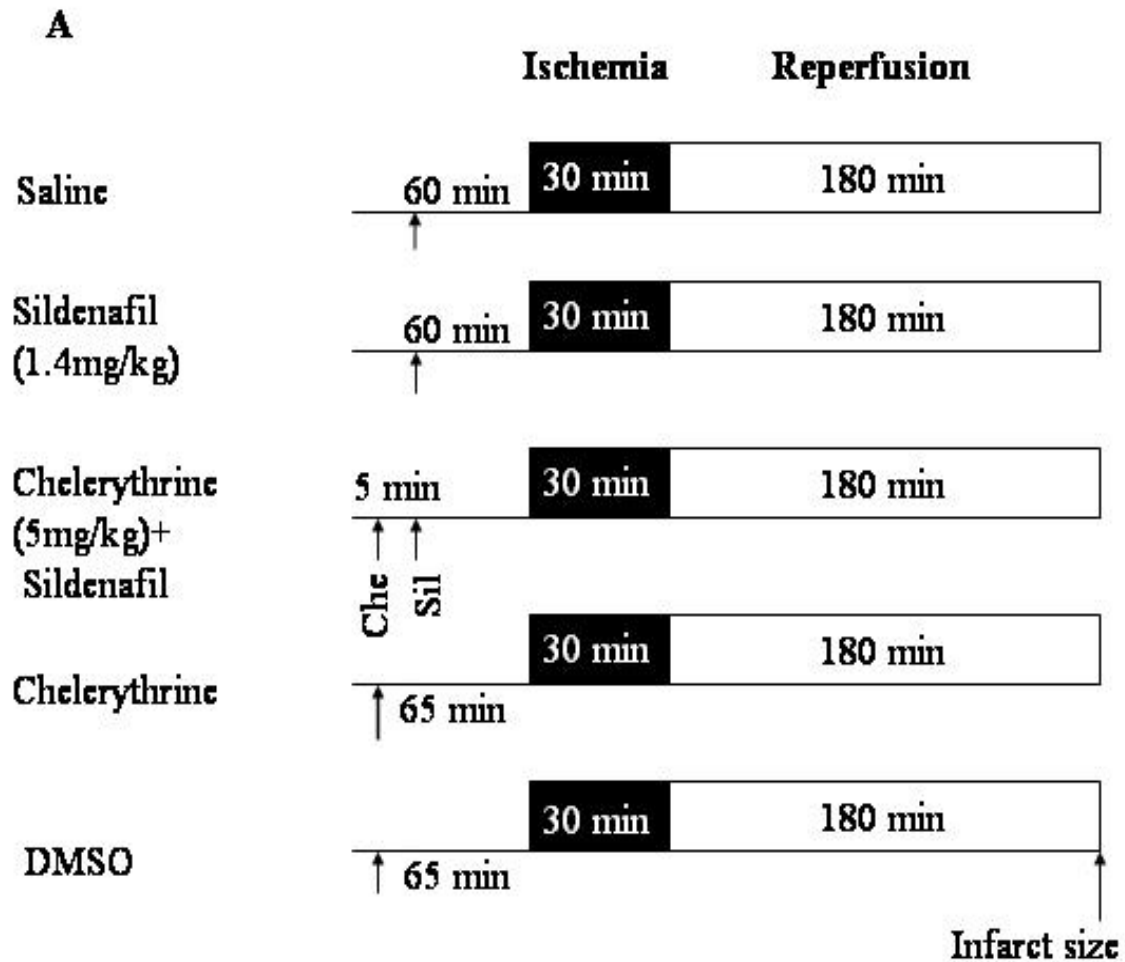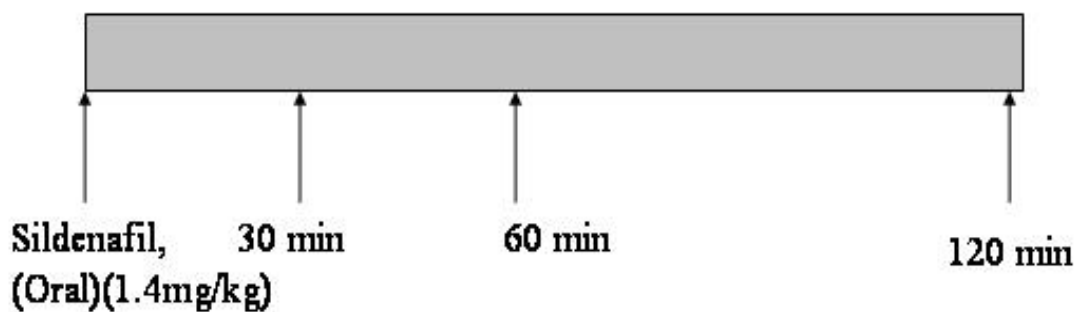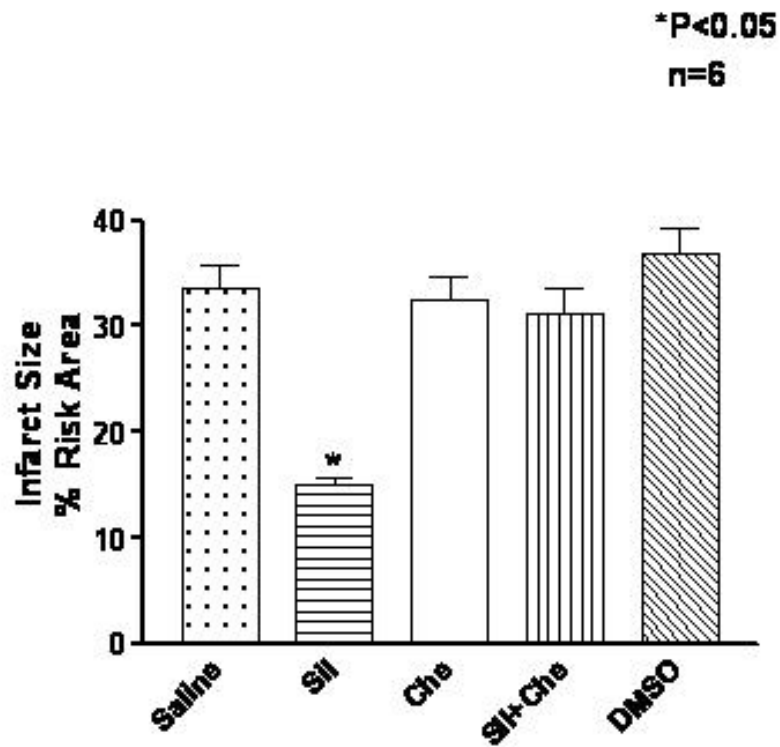
CHAPTER 5

Sildenafil Citrate (Viagra) Induces Cardioprotective Effects Following Ischemia-Reperfusion Injury in Infant Rabbits

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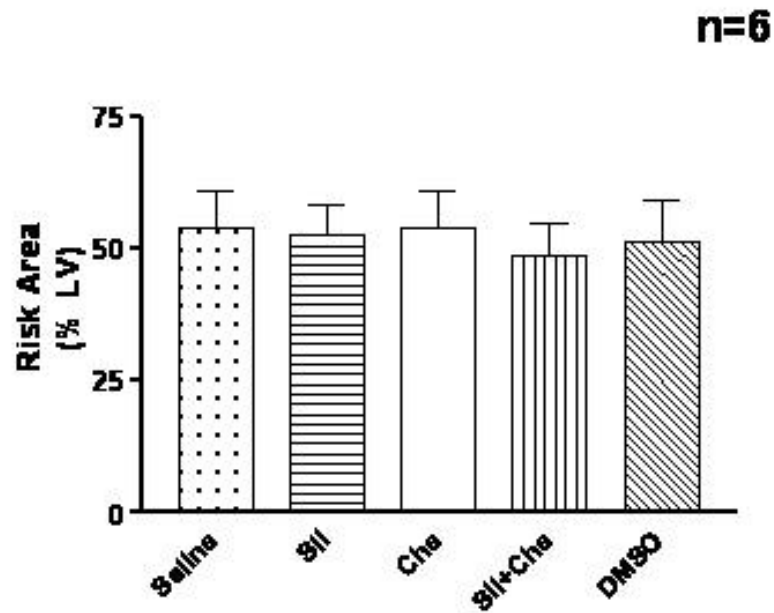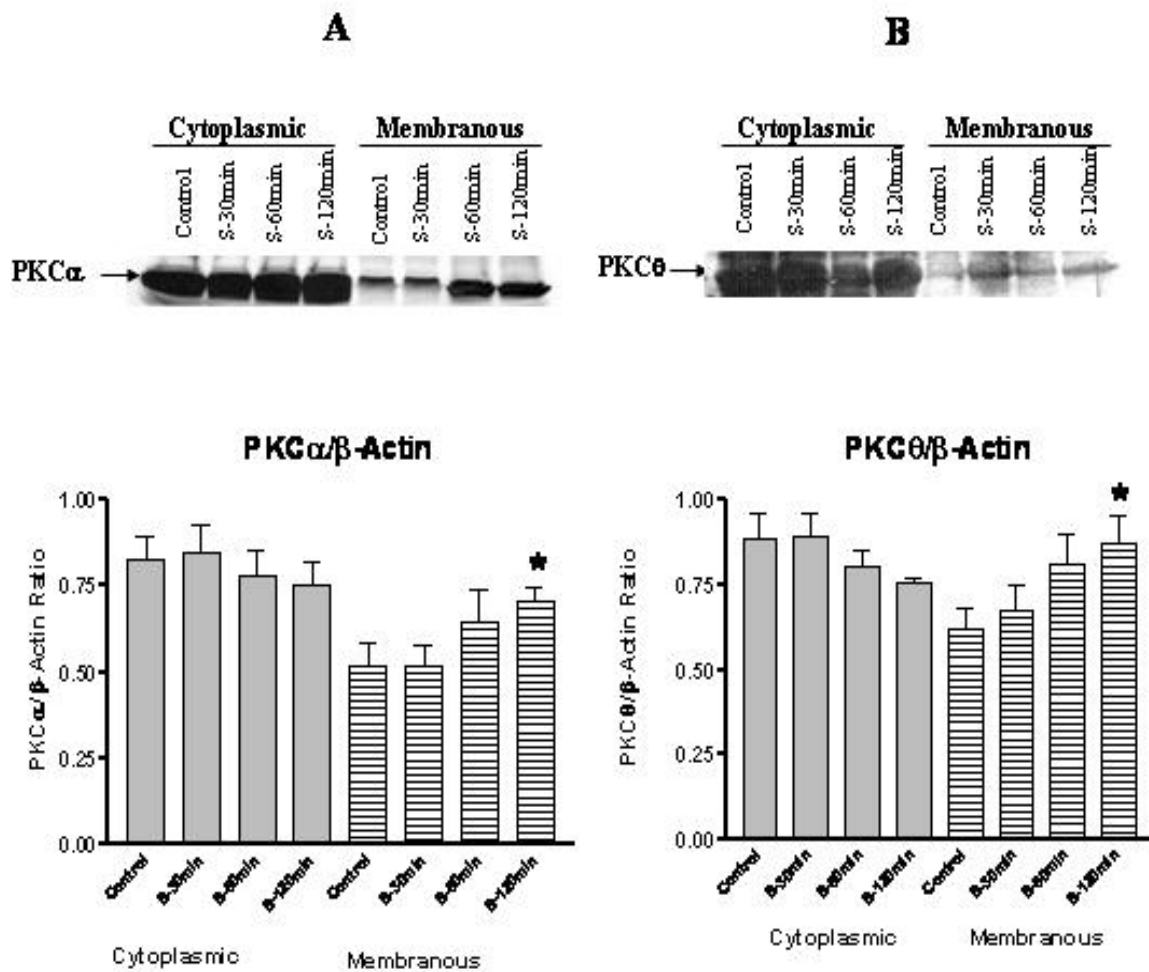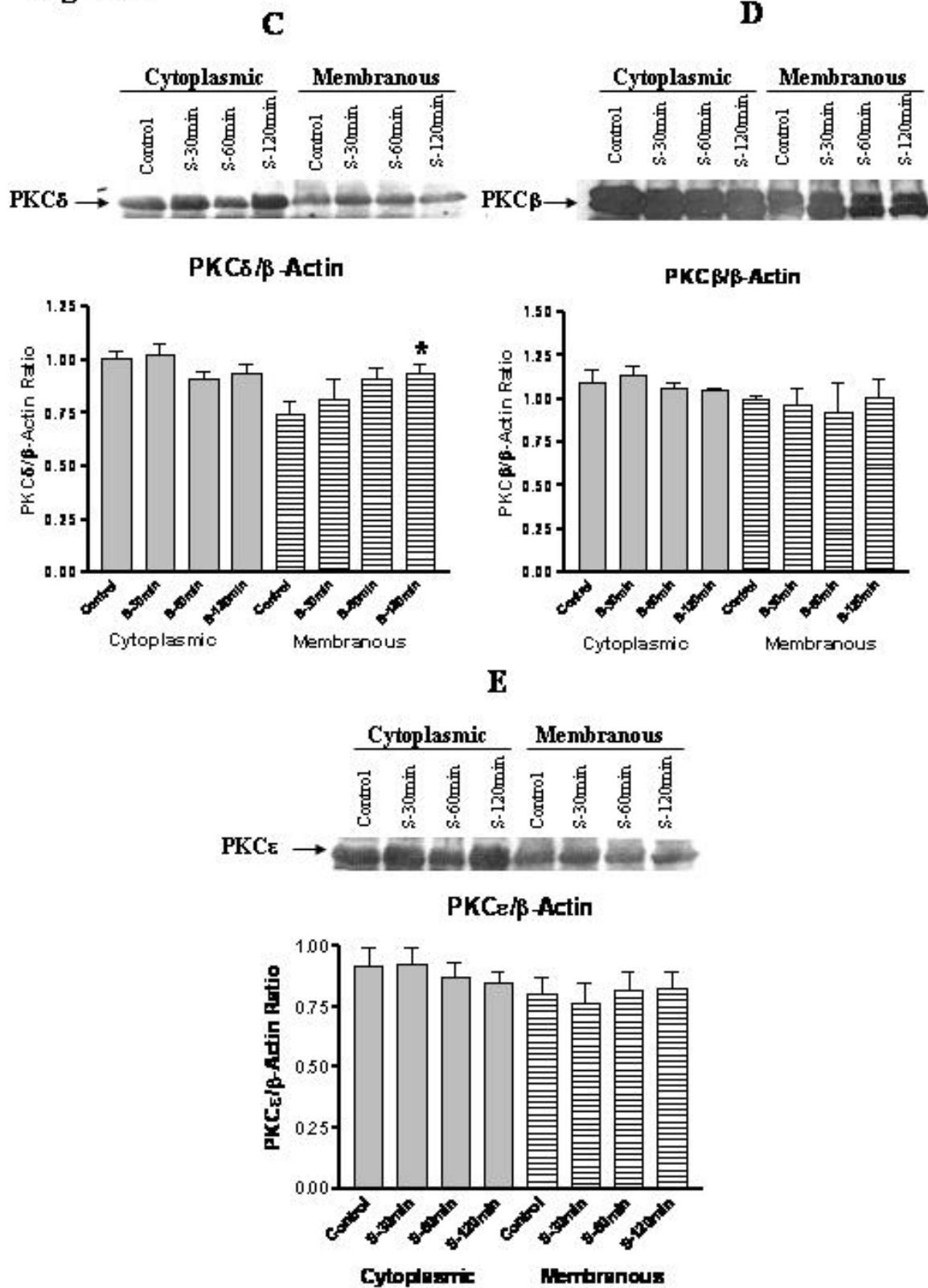Division of Cardiology, Department of Medicine [F.S., R.O., E.C., R.C.K.],
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ABSTRACT

Infants undergoing surgery for congenital heart disease are at risk for myocardial ischemia during cardiopulmonary bypass, circulatory arrest, or low flow states. The purpose of this study was to demonstrate the effects of sildenafil, a selective phosphodiesterase-5 (PDE-5) inhibitor on myocardial functional improvement and infarct size reduction during ischemia/reperfusion injury in infant rabbits. Infant rabbits (aged 8 weeks) were treated with sildenafil citrate (0.7 mg/kg IV) or normal saline 30 minutes prior to sustained ischemia for 30 minutes and reperfusion for 3 hours. Transesophageal echocardiography (TEE) was utilized to assess left ventricular cardiac output (LVCO) and aortic Velocity Time Integral (VTI). Following ischemia/reperfusion, risk area was demarcated by Evan's blue dye and infarct size determined by computer morphometry of triphenyltetrazolium chloride stained sections. The sildenafil-treated group had

preservation and elevation in LVCO (143% of baseline, $p<0.05$) and an elevated aortic VTI (145% of baseline, $p<0.05$) following 30 minutes of ischemia compared to the control group LVCO (72% of baseline, $p<0.05$) and aortic VTI (73% of baseline, $p<0.05$). This is a statistically significant increase in LVCO and aortic VTI in the sildenafil group compared to controls (n=6/group, $p<0.05$). The sildenafil treated group had significant reduction in infarct size (15.5 ± 1.2 versus 33 ± 2.3 in the saline group, % risk area, mean ± SEM, n=10-15/group, $p<0.05$). For the first time, we have shown that sildenafil citrate promotes myocardial protection in infant rabbits as evidenced by post-ischemic preservation and elevation in LVCO and aortic VTI and reduction in infarct size.

Abbreviations

PDE-5, phosphodiesterase-5

TEE, transesophageal echocardiography

LVCO, left ventricular cardiac output

VTI, velocity time integral

TTC, 2,3,5-triphenyltetrazolium chloride

LAD, left anterior descending

LV, left ventricle

LVOT, left ventricular outflow tract

HR, heart rate

MAP, mean arterial pressure

SVR, systemic vascular resistance

Each year, more than 25,000 children undergo corrective surgery for congenital heart disease. Early surgical intervention is important to promote more normal development. Infants undergoing surgery for congenital heart disease are at risk for myocardial ischemia during cardiopulmonary bypass, circulatory arrest or low flow states (1). Less is known about the tolerance of the infant myocardium to ischemia compared to the adult, and cardiac reserves are more limited in the infant (2). Although there is evidence that the infant myocardium may be more resilient to metabolic or ischemic injury compared to adults, the infant heart responds quite differently to cardiovasular drugs, stress, and changes in hemodynamics (3,4). Although hypothermia combined with pharmacologic cardioplegia protects the globally ischemic adult heart, this benefit may not extend to infants. For example, poor post-ischemic recovery of function and increased mortality may result when this method of myocardial protection is used in children (5). Therefore, there is a need to develop novel pharmacological approaches to protect the infant hearts from ischemia/reperfusion injury.

Brief episodes of ischemia protect the myocardium from more prolonged periods of ischemia, a phenomenon called ischemic preconditioning (6). A variety of other stimuli, such as hypoxia, thermal stress, pharmacologic agents, and endogenous triggers of preconditioning such as nitric oxide and adenosine have also been shown to induce cardioprotective effects in several animal species (7-10). Also, recent studies from our laboratory have shown that sildenafil citrate (Viagra), a selective phosphodiesterase-5 (PDE-5) inhibitor, induces powerful preconditioning-like protective effects in the ischemic heart (11). However, it is not known whether sildenafil exerts similar protective effects

against ischemia/reperfusion injury in the infant rabbit hearts as well. The purpose of this study was (a) to show if pretreatment of infant rabbits with clinically relevant dose of sildenafil improves post-ischemic myocardial function and (b) to demonstrate if this drug reduces myocardial infarct size following ischemia/reperfusion. We used the model of coronary artery occlusion and reperfusion in infant rabbits, which is similar to our previously described adult rabbit model of myocardial infarction (11). This model may be applicable in pediatrics, and especially in pediatric cardiovascular surgery where there may be periods of ischemia/reperfusion injury. Also, for the first time, we used 2-dimensional (2D) and Doppler transesophageal echocardiography (TEE) for the estimation of left ventricular cardiac output (LVCO) and aortic velocity time integral (VTI) in this model.

METHODS

Animals. Eight-week-old male New Zealand White rabbits with mean weight of 2.0 kg (range 1.3 kg-2.6 kg) were used for the study. We chose Eight-week-old rabbits because they are similar in size to human neonates or young infants who undergo surgery for congenital heart disease early on in life. We believe this model to be different from the adult rabbit model as well as helpful and similar to young human infants because of similar size, presence of thymic tissue (and thus not having had undergone significant stress yet), and being prepubertal. We thus believe that the physiology and responses to stress and ischemia may be similar to that of human infants.

Study Protocol. Anesthesia and Infarction protocol are similar to those in Chapter 2.

TEE. Transesophageal echocardiography, using a 10 Fr AcuNav diagnostic ultrasound probe, was performed at baseline, following the 30 minute period of ischemia, and after 3 hours of reperfusion in both the control and sildenafil groups. Standardized 2D imaging in a long axis view of the left ventricle (LV) to show LV inflow across the mitral valve and left ventricular outflow tract (LVOT) was obtained (12) (Fig. 1). Aortic flow Doppler across the aortic valve was performed in a long axis view of the LV and LVOT to obtain LVCO (Fig. 2). The standard equation: Mean Velocity (cm/s) x flow area (cm^2) x 60 (s/min), where Mean Velocity (cm/s) = VTI (in cm/beat) ÷ RR interval in s/beat, was used to obtain LVCO expressed in mL/min (12,13). Laminar Doppler flow across the aortic valve confirmed the absence of aortic stenosis. Color Doppler assessment was made of both the aortic and mitral valves again in the long axis view at baseline, after the ischemic period, and following 3 hours of reperfusion for the presence or absence of mitral or aortic regurgitation. Subjective functional assessment was also made following ischemia and reperfusion to demonstrate at least left ventricular apical diminished contractility to confirm infarction.

Measurement of Infarct Size. The same methodology was used here as previously dicussed in Chapter 2.

RESULTS

Animals and Exclusion. A total of 34 rabbits were used in the study; 20 in the sildenafil group and 14 in the control group. 2 rabbits in the control group died before completion of the 3 hour reperfusion period due to hypotension, arrhythmias and acidosis. TEE was performed on 12 rabbits; 6 in the sildenafil group and 6 in the control group. 2 rabbits in the control group did not undergo TEE following 3 hours of reperfusion due to technical difficulties with the TEE machine. A total of 25 rabbit hearts were examined for infarct size, 15 in the sildenafil group and 10 in the control group. The 9 rabbit hearts that did not stain well were excluded from the study.

Hemodynamics. Changes in hemodynamics are summarized in Table 1. The rabbits treated with sildenafil had a 34% decline in mean arterial pressure (MAP) and 8% increase in heart rate (HR) following drug administration as compared to the controls ($p < 0.05$). However, MAP and HR were comparable in both groups prior to the ischemic period. Following 30 minute ischemia, MAP and HR were also comparable in both the groups although a significant decline in MAP as compared to the baseline values was observed. This drop in MAP persisted through 3 hours of reperfusion, which represented a 19% and 18% decrease for the sildenafil and the control group respectively, compared to baseline values ($p < 0.05$).

TEE. Both the control and sildenafil-treated groups had comparable LVCO and aortic VTI at baseline. The controls had a decline in LVCO and aortic VTI immediately following the 30 minute period of ischemia (28% and 27% lower than baseline values, respectively, $p<0.05$), whereas the LVCO and aortic VTI increased in the sildenafil group following ischemia (43% and 45% higher than baseline values, respectively, n = 6 per group, $p<0.05$). Both groups, however, had significant decline in LVCO following 3 hours of reperfusion (54% of baseline in the sildenafil group, $p<0.05$, and 62% of baseline in the control group, $p<0.05$), and were not statistically significantly different from each other (n = 4-6 per group). Both groups demonstrated a decrease in aortic VTI following 3 hours of reperfusion. However, this decline was only statistically significant in the control group compared to baseline values. Changes in LVCO and aortic VTI are shown in Figure 3. None of the rabbits had aortic stenosis or developed aortic regurgitation for the duration of the study. Both the control and sildenafil groups demonstrated a comparable amount of mitral regurgitation (no more than mild) following ischemia/reperfusion, and none of the rabbits had baseline mitral regurgitation.

Infarct Size. Figure 4 shows risk area (% LV) and infarct size expressed as the % of risk area. Pretreatment with sildenafil resulted in a decrease in the infarct size from 33 ± 2.3 in the control group to 15.5 ± 1.2 in the sildenafil treated rabbits, a 45% reduction compared to controls ($p<0.05$). A similar trend in the changes in infarct size was observed when expressed as % of LV (not shown). Similarly, the differences between the risk areas were not statistically significant between the two groups. These data suggest that changes in the

infarct size observed in the two groups was not related to the % of the area of LV occluded by our technique.

DISCUSSION

The use of sildenafil for treatment of erectile dysfunction by many patients with cardiovascular disease has resulted in a tremendous interest in the cardiovascular properties of the drug (14). Recent studies in rats have shown that PDE-5 inhibition with sildenafil attenuated the rise in pulmonary artery pressure and vascular remodeling when given before chronic exposure to hypoxia and when administered as a treatment during ongoing hypoxia-induced pulmonary hypertension (15). Clinical investigations in patients with pulmonary arterial hypertension have also shown that sildenafil therapy may be of benefit in patients receiving long-term infusion of epoprostenol (16,17). Recently we reported that administration of sildenfil induced cardioprotection as indicated by significant reduction in infarct size when compared to controls (11,18). In addition, we showed that sildenafil induced delayed cardioprotective effect in the mouse heart through upregulation of iNOS and eNOS (19). The hypothesis behind these studies was that the vasodilatory action of sildenafil could potentially release endogenous mediators of preconditioning such as adenosine, bradykinin, or NO. One or more of these mediators may trigger a signaling cascade leading to activation of protein kinase C (20) and opening of the mitoK$_{ATP}$ channel resulting in acute and delayed cardioprotective effects (11). In the present study, we have shown that sildenafil citrate also induced cardioprotection against

ischemia/reperfusion injury in infant rabbits. This is evident from preservation of post-ischemic cardiac output and significant reduction in infarct size.

Hemodynamics. Sildenafil citrate had a direct effect on hemodynamics by causing transient hypotension, which may have resulted in increased heart rate either directly or indirectly. Both groups developed the same degree of hypotension following ischemia even though the sildenafil-treated group had increased LVCO and aortic VTI. This may reflect sildenafil's effect on the lowering of systemic vascular resistance (SVR). Since hypotension was again observed following the 3 hour reperfusion period in both groups, and LVCO was comparable even though infarct size was reduced in the sildenafil group, it is likely that LVCO and aortic VTI may be predominantly related to SVR. We believe that sildenafil's effect on SVR, which is not directly measured by pressure and heart rate, caused such a profound change and increase in cardiac output. Thus, it is also possible that the additional benefits of sildenafil may be short-lived. Our results suggest that multiple mechanisms may be involved in myocardial preservation with sildenafil infusion. This may include a preconditioning-like effect as well as a direct effect on hemodynamics. The drop in MAP in both groups following the 3 hour reperfusion period may reflect the fact that all rabbits were under general anesthesia for the duration of the experiment. The rabbits did receive 50cc/hr of normal saline (~25cc/kg/hr), which is the approximate equivalent of several fluid boluses as given to human infants, although infused more slowly. Since urine output was maintained throughout the experiment, we believe that renal function was preserved, and any excessive volume infused would have been accounted for by adequate urine output. We would have expected hypertension and

bradycardia, or similar echocardiographic findings of increased output due to increased preload in both groups if volume had been an issue. Thus it is unlikely that volume infusion alone led to hemodynamic deterioration in both groups after the reperfusion period.

Functional assessment. In the present study, we used 2D and Doppler echocardiography for the estimation of LVCO and aortic VTI. This may be a better marker for preserved or increased cardiac output. The use of TEE in the infant rabbit provided a long axis view of the LV and LVOT to make these calculations. This view also showed the mitral valve and a selective plane of the left ventricular myocardium. LVCO was preserved in the sildenafil treated group mainly because of increased aortic VTI. Aortic VTI, when multiplied by cross-sectional area, gives a volume measurement, or in this case left ventricular stroke volume (13,21). When this is multiplied by heart rate, it gives left ventricular cardiac output (13,21). Heart rate was similar in both groups following ischemia and reperfusion, so can not be assumed to be the major determinant. In addition, the cross-sectional area of the aortic valve annulus remained essentially unchanged as well. LVCO was elevated in the sildenafil group compared to its baseline values and compared to the control group following the ischemic period. Since cardiac output is a function of preload, afterload, and contractility, this demonstrates the beneficial effects of sildenafil in preserving myocardial function through its effect on hemodynamics and SVR (afterload). We believe that sildenafil's effect on SVR, which is not directly measured by pressure and heart rate, caused such a profound change and increase in cardiac output. We can not assume this is due to myocardial contractility alone (since this was not directly measured). It is likely

that sildenfil's role in opening of mitoK$_{ATP}$ channels contributed to less myocardial necrosis, and subsequently enhanced contractility, although this does not explain the decline in function in both groups following the 3 hour reperfusion period. Perhaps to a lesser extent, sildenafil inhibited the degradation of cAMP (by its minor role in inhibiting PDE-3), and subsequently led to accumulation of ATP and improvement in contractility. In addition, it is possible that sildenafil may cause coronary vasodilation and thus lead to enhanced local and selective myocardial perfusion following an ischemic period, and again, improvement in contractility. The decline in cardiac output following 3 hours of reperfusion in both groups suggests that functional protection with sildenafil may be short-lived, and again, predominantly related to SVR. It may also be related to undefined actions of the drug itself that have yet to be determined.

Infarct Size. In the present study, we observed significant decrease in the infarct size in the sildenafil treated group as compared to control rabbits. However the degree of infarct reduction was 53 % in the infant model as compared to 68 % in the adult rabbits (11) during acute pretreatment with sildenafil. The drug provided 75 % reduction in infarct size 24 hrs after treatment in the mouse model of global ischemia and reperfusion (19). The reason for these variations is not clear although it may be related to the physiologic differences in the resistance of the infant myocardium to ischemia or stress versus the difference in response of the infant myocardium to cardiovascular drugs. Although the infant myocardium may be more tolerant of ischemia only, its response to stress,

hemodynamic changes, and pharmacologic agents may be very different, thus rendering the myocardium more susceptible to injury.

Study limitations. When using Doppler echocardiography to calculate LVCO, there are several fundamental assumptions. First, that flow is laminar and organized; second, that the velocity profile is uniform across the aortic valve outlet; third, that the point of outflow used to calculate vessel cross-sectional area is circular; and fourth, that the point of measurement of cross-sectional area remains relatively constant without significant changes with hemodynamics (13,21). We made every attempt to record Doppler tracings with the Doppler beam parallel with flow in the vessel and just above the aortic valve annulus, as this is the point at which maximal flow velocity should theoretically occur (13). We also measured the cross-sectional area at the aortic valve annulus, as this area should be the flow-limiting point and most constant in relation to changes in hemodynamics (13). Since LVCO is preload dependent, the presence of mitral regurgitation or aortic regurgitation may potentially have an effect on cardiac output. Although we did not account for the volume of mitral regurgitation, we did assess for its presence or absence. Since this amount was subjectively similar in both groups, we assumed that LVCO would not be substantially affected in either group. Neither group developed aortic regurgitation. Our study is also limited to a single 2D image of the long axis of the left ventricle. This view is not able to assess all segments of the left ventricular myocardium to quantitate regional wall motion abnormalities. Although we were able to

subjectively determine diminished apical myocardial contractility following infarct, we limited our study to the assessment of aortic VTI and LVCO.

Clinical implications. For the first time, we have shown that sildenafil treatment resulted in post-ischemic early improvement in cardiac output during ischemia/reperfusion in infant rabbits. In addition, the drug caused significant reduction in infarct size following ischemia/reperfusion. Further studies are needed to determine the effects of hypotension alone, as well as duration and timing of drug administration on sustained preservation of myocardial function during reperfusion or days later during recovery. Since less is known about the optimum method of myocardial preservation in infants compared to adults, there is impetus for studying and developing different strategies for improved surgical outcome. In addition, since postoperative management of infants with congenital heart disease is complicated by various physiologic differences and responses to ischemia, stress, and changes in hemodynamics, it is important to devise new techniques for ideal ICU care. It is possible that sildenafil citrate may be clinically important in protection of the heart in the setting of cardiac surgery employing cardiopulmonary bypass, circulatory arrest, or low flow states in infants with congenital heart disease.

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FIGURE LEGENDS

Figure 1. Still frame 2D transesophageal echocardiogram of LVOT. LA=left atrium, MV=mitral valve, LV=left ventricle, AV=aortic valve, AO=aorta, RV=right ventricle.

Figure 2. Still frame Doppler across aortic valve, velocity vs time.

Figure 3. LVCO and aortic VTI following sildenafil infusion during ischemia/reperfusion. n = 4-6 per group. *$p<0.05$, [†]$p<0.05$ compared to baseline values.

Figure 4. Bar diagram illustrating infarct size (% risk area) in controls vs the sildenafil group compared to risk area (% left ventricle). n = 10-15 per group. *$p<0.05$.
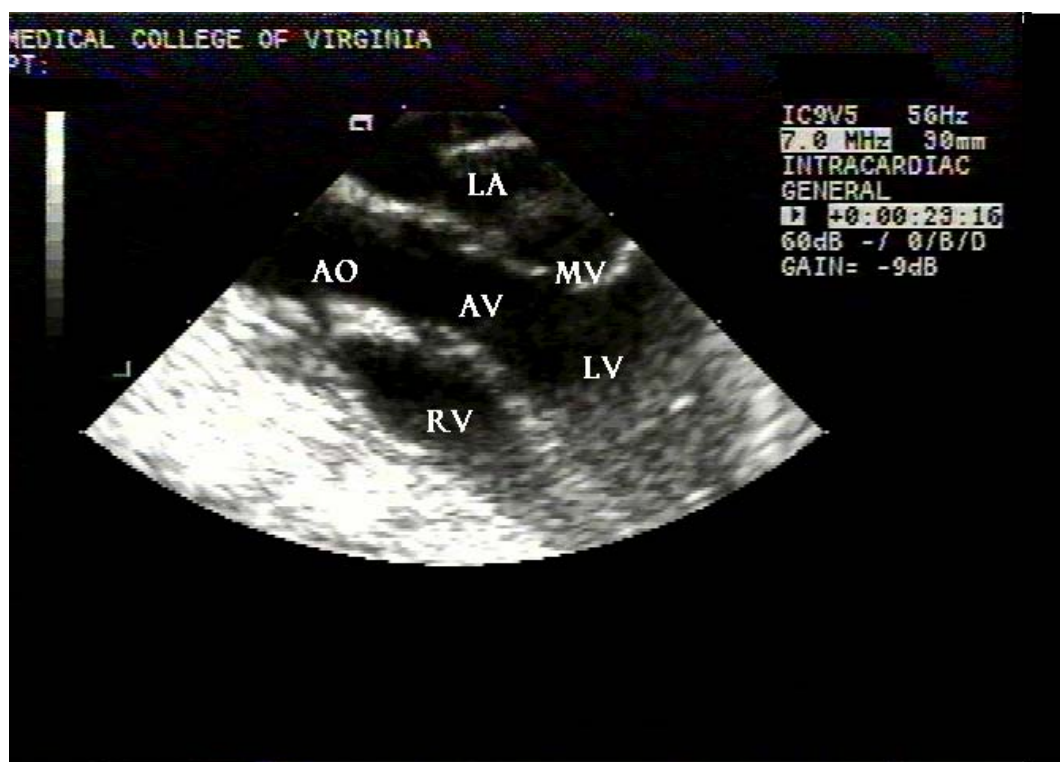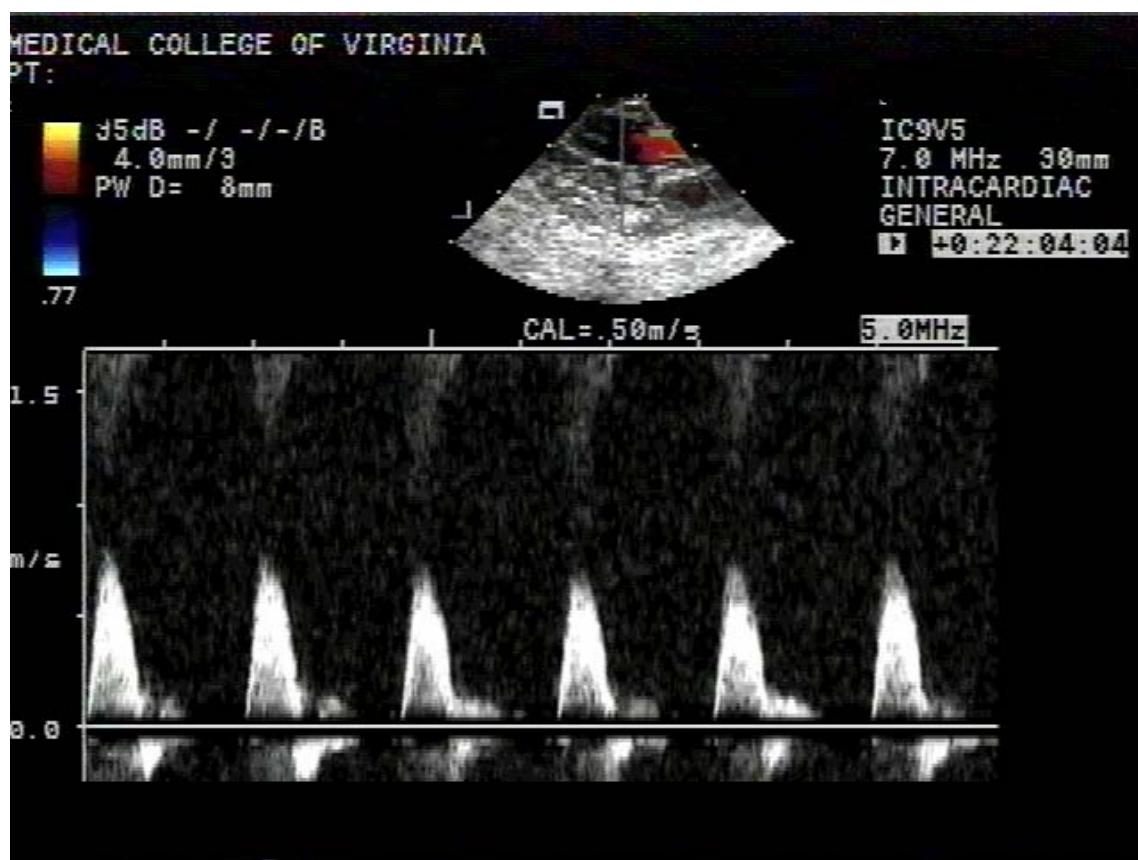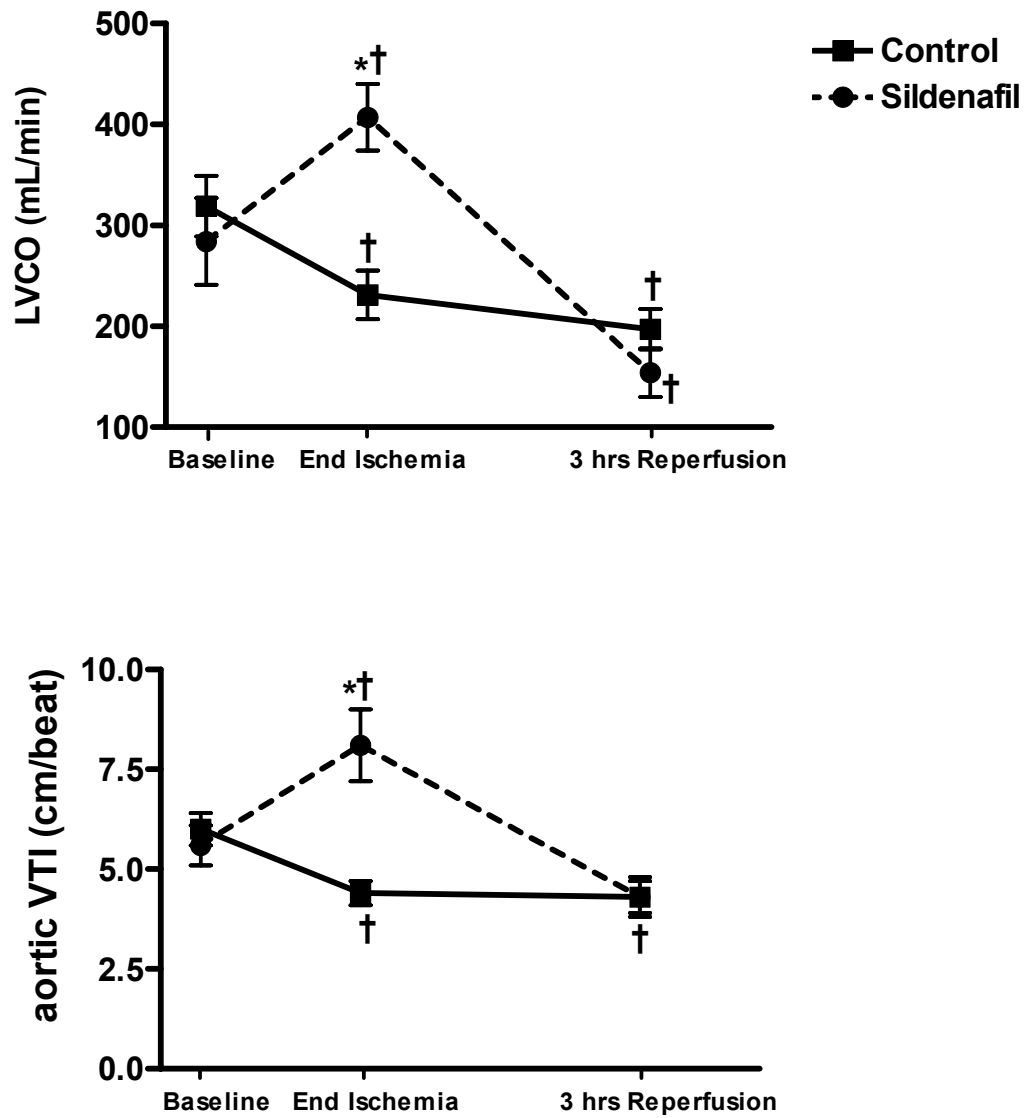
Figure 1

Figure 2

Figure 3

Figure 4
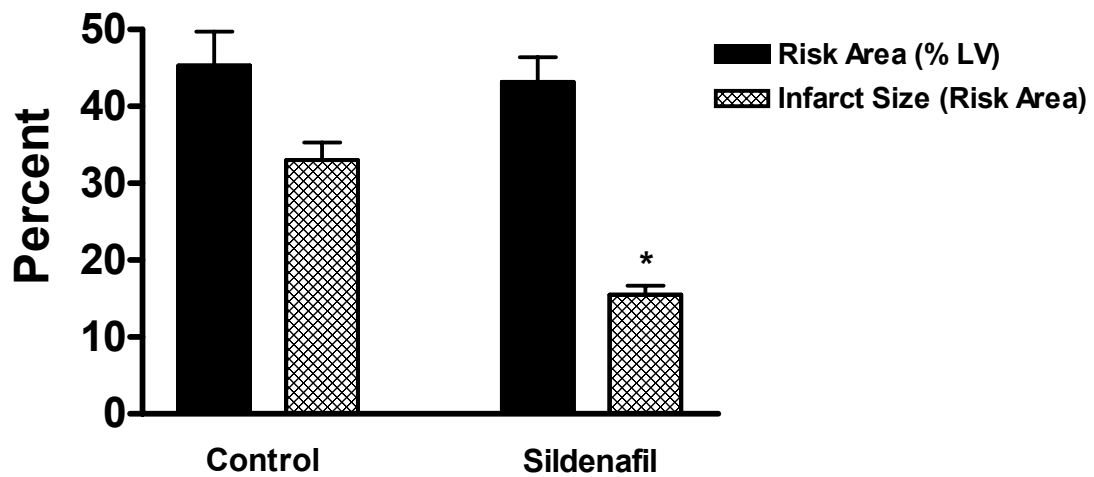
Table 1. Hemodynamic Data During Ischemia/Reperfusion

	Baseline	After Saline/Drug	Preischemia	End Ischemia	Reperfusion Time (Hour)		
Cntrl					1	2	3
MAP	85.4± 3.7	84.3± 3.4	80.5± 3.8	73.1± 4.3§	75.1± 3.4	75.3± 3.4	69.7± 3.5§
HR	213.1± 6.6	213.8± 7.1	212.1± 6.9	206.1± 6.9	188.5± 7.4	175.3± 7.2	169.7± 8
SIL							
MAP	84.8± 3.7	55.9± 2.5*	74± 3.7†	70.2± 4‡	71.5± 3.2	69.2± 2.8	69.1± 2.8‡
HR	218.3± 6.6	236.1± 4.6*	211.8± 3.8†	202.2± 5	180.2± 6.9	174.1± 7.2	167.8± 7.1

Values expressed as means ± SEM. MAP=mean arterial pressure in mmHg; HR= heart ratein beats per minute. n = 12-20 per group. *p<0.05 vs controls; †p>0.05 vs controls; ‡p>0.05 vs controls but <0.05 vs baseline; §p<0.05 vs baseline.

CHAPTER 6

Sildenafil Induces Delayed Preconditioning against Myocardial Infarction via iNOS Upregulation by Phosphorylation of ERK in Mouse Heart

Background: We previously demonstrated that sildenafil induces delayed preconditioning following global ischemia/reperfusion in the mouse heart via iNOS dependent pathway (Chapter 3). In the current study, we investigated the role of MAP kinases in sildenafil-induced cardioprotective signaling.

Methods and Results: Adult male ICR mice were pretreated (i.p.) with either sildenafil (0.71 mg/kg, equivalent to 50 mg dose for 70 kg patient) or volume-matched saline. Selective inhibitors of p38 MAPK (SB203580, 1 mg/kg, i.p.) or MEK1 and 2 (PD 98059, 1 mg/kg, i.p.) were administered 30 min before sildenafil. The hearts were isolated 24 hours later and subjected to 20 min global ischemia and 30 min of reperfusion in Langendorff mode. Infarct size was measured by computer morphometry of TTC stained sections. Post-ischemic myocardial infarct size (Mean±SEM; % of risk area) was reduced in mice treated with sildenafil (6.0±1.2) versus saline group (27.6±3.3; $P<0.05$; n=6/each). SB203580 did not block this protective effect (8.2±1.6 vs. 6.0±1.2 with sildenafil alone, $p>0.05$). In contrast, PD98059 treatment abolished the infarct limiting effect of sildenafil (22.18±2.76, $p<0.05$). Sildenafil caused increased phosphorylation of ERK1/2 but not p38 MAPK within 30 min after treatment. Furthermore, western blot analysis showed an elevation in iNOS protein levels 24 hrs after treatment with sildenafil, which was abolished by

PD98059. No significant changes in ventricular contractile function and heart rate were observed in sildenafil-treated group.

Conclusion: We conclude that the sildenafil-induced delayed protection against global ischemia/reperfusion is mediated by signaling pathway involving phosphorylation of ERK and the subsequent upregulation of iNOS.

INTRODUCTION

Sildenafil citrate (Viagra) is the first oral agent approved for treatment of erectile dysfunction in men. It is a selective inhibitor of phosphodiesterase-5 (PDE-5), an enzyme that catalyzes the breakdown of a potent smooth muscle relaxing agent cyclic guanosine monophosphate (cGMP). Sildenafil has been shown to cause moderate decrease in systolic and diastolic pressure because of the inhibition of PDE-5 in smooth muscles in the vascular bed. We hypothesized that such mild vasodilatory effect of sildenafil in coronary vasculature could potentially release vasoactive agents such as adenosine or bradykinin, and demonstrated the role of sildenafil in increasing levels of iNOS protein which in turn upregulate NO that may trigger preconditioning-like protective effect in the heart. Moreover, we recently showed that sildenafil caused acute and delayed protection against ischemia/reperfusion injury *in vivo* in the heart which was dependent on the opening of mitoK$_{ATP}$ channel [88]. These studies were performed in an *in situ* rabbit model where the hearts were subjected to coronary artery occlusion followed by reperfusion. We also illustrated that sildenafil induced a powerful delayed cardioprotevtive effect against global

ischemia/reperfusion injury via iNOS-dependent pathway in an isolated perfused mouse heart model [90]. However, the signaling pathway(s) that lead to such cardioprotective effect are currently under investigation.

Recent studies suggest an important role of the MAP kinase family particularly p38 in the early phase of ischemic preconditioning (IPC) [20], although the results have been controversial [92]. Direct activation of p38 by anisomycin mimicked IPC and delayed cardioprotection in the isolated perfused hearts [93,46]. The activation of p38 has also been suggested to be the trigger of delayed preconditioning induced by adenosine. In the MAP kinase family, the p44/p42-MAPK (Erk1/2, where Erk is extracellular-signal-regulated kinase) cascade appears to mediate cell growth and survival signals in many cell types. ERK activation has been shown to protect cardiomyocytes from oxidant stress [94]. Sustained activation of p44/p42-MAPK during simulated 'reperfusion' following sublethal simulated 'ischemia' mediates preconditioning in cardiomyocytes independently of transient activation of p38-MAPK. However there are no studies available implicating the role of MAP kinases in sildenafil-induced cardioprotection. The goal of the present investigation was to 1) to show if p38 MAPK and ERK 1 and 2 phosphorylation plays role in the sildenafil-induced cardioprotection, and 2) to investigate the role of MAPK phosphorylation in the sildenafil-mediated increase in iNOS and eNOS protein levels.

MATERIALS AND METHODS

Langendorff Isolated Perfused Heart Preparation

The same experimantal protocol was used as in Chapter3.

Study Protocol

Mice were randomly assigned into one of the following groups:

Group I: (saline control, n=6): Mice received 0.9 % saline, *i.p.*;

Group II: (Sildenafil, n=6): Viagra tablets were crushed and dissolved in saline. This preparation was given as 0.71 mg/kg *i.p.* bolus, approximating, on a mg/kg basis, the clinical dose of 50 mg administered to a 70 kg patient;

Group III: SB203580 + Sildenafil (n=6): SB203580 (1.0 mg/kg) was injected 30 min prior to sildenafil treatment as in Group II;

Group IV: *SB203580* + Saline (n=6): treatment with SB203580 alone;

Group V: PD 98059 (1.0 mg/kg) was injected 30 min prior to sildenafil;

Group VI: PD 98059 + Saline (n=6): pretreatment with PD 98059 alone;

Twenty-four hours later, the hearts were isolated and following a 30 min stabilization period, the hearts were subjected to 20 min no-flow normothermic global ischemia and 30 min reperfusion.

Phosphorylation of p38, ERK 1 and 2: Four animals were pretreated with 0.15 ml saline or 0.71mg/kg sildenafil (i.p., n=2 per group). Te hearts were removed, ventricular samples collected 30 min after treatment and immediately frozen in liquid nitrogen. The samples were ground into fine powder in liquid nitrogen after cell lysis in 1 ml of RIPA buffer (Upstate) containing: 5omM Tris-HCL, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1mM EDTA and protease inhibitors (1nM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin and μg/ml aprotinin) and a phosphatase inhibitor (1 mM sodium orthovanadate). The mixture was homogenized and centrifuged at 6,000 *g* for 10 min. The supernatant was then collected and protein concentration was measured.

For immunoprecipitation, 300 μg of the sample protein were incubated with 5 μg of anti-p38 (C-20, Santa Cruz) or anti-ERK rabbit polyclonal antibody (C-14, Santa Cruz) in 500 μl of RIPA buffer for 3 h at 4°C. 35 μl of protein A/G agarose beads (Santa Cruz) were then added to the sample and mixed for another 60 min. The agarose beads containing the immunocomplexes were pelleted by centrifugation for 5 min (1000 *g*) at 4°C, and the supernatant was carefully aspirated and discarded. The beads were washed 3 times with RIPA buffer and 1 time with PBS by repeating the centrifugation step described above. The beads were then resuspended in 50 μl of electrophoresis sample buffer. To dissociate the immunocomplexes from the beads, the sample was boiled for 5 min and centrifuged again, and the supernatant was collected. Subsequently 20 μl of immunoprecipitated supernatant were separated by SDS-PAGE (10% polyacrylamide) and transferred to a polyvinylidene difluoride membrane (Bio-Rad) via tank transfer for 2 h at 190 mA. After the membrane was blocked with milk solution [5% nonfat dry milk in Tris-buffered saline

(TBS) with 0.05% Tween-20 (TBST)] for 1 h, it was probed with a mouse monoclonal antibody for either phosphorylated p38 MAPK (p-p38, D-8; Santa Cruz) or phosphorylated ERK (p-ERK, E-4, Santa Cruz); or total p38 (A-12, Santa Cruz) or ERK-2 (D-2, Santa Cruz) [both diluted 1:500 in milk with TBST] for 2 h. After the membrane was washed with TBST, it was incubated with an anti-mouse horseradish peroxidase-linked antibody (diluted 1:2,000 in milk solution with TBST; Amersham) for 1 h. The membrane was washed with TBST four times (for a total of 30 min) and then incubated using a chemiluminescence kit (Amersham) before being exposed to X-ray film. The bands were quantified via densitometric scanning.

Western Blot Analysis: In brief, triplicate heart samples were collected 24 hours after saline , sildenafil, PD98059 followed by sildenafil 30 min later, or PD98059 injection and homogenized in ice-cold RIPA buffer (Upstate Biotechnology). The homogenate was centrifuged at 10,000 g for 10 min under 4°C, and supernatant was recovered as the total cellular protein. 60 µg total protein from each sample was separated by SDS/PAGE on 10% acrylamide gels and transferred to a PVDF membrane, and then blocked with 5% non-fat dry milk in TBS. The membrane was subsequently incubated with a rabbit polyclonal antibody (Santa Cruz; dilution 1:500) reacting specifically to iNOS, eNOS, or actin (for normalizing loading of protein). The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, 1:1,000 dilution). The membranes were developed using enhanced chemiluminescence and exposed to x-ray film.

For quantifying protein expression, the optical density for each Western blot band was scanned and analyzed with a densitometric system (Bioquant 98).

RESULTS

Cardiac Hemodynamics and Contractile Function:

There was no significant difference in the basal functional parameters (*i.e.* developed force, rate-force product, and resting tension) between the groups after 20 min stabilization.During post-ischemia also; there were no significant differences in the rate force product (Figure 1). Post-ischemic coronary flow was not significantly different among the groups except that it was slightly improved in the sildenafil-treated mice.

Myocardial Infarction:

Post-ischemic myocardial infarct size (Mean±SEM; % of risk area) was reduced in mice treated with sildenafil (6.0±1.2) versus saline group (27.6±3.3; P<0.05; n=6/each). SB203580 did not block this protective effect (8.2±1.6 vs. 6.0±1.2 with sildenafil alone, p>0.05). In contrast, PD98059 treatment abolished the infarct limiting effect of sildenafil (22.18±2.76, p<0.05). There was no significant difference in the infarct size between the controls and the animals treated with DMSO, PD98059 and SB203580.

Phosphorylation of p38 and ERK:

Ventricular tissue was harvested after 30 minutes of treatment with sildenafil. Phosphorylation of p38-MAPK and p44/p42-MAPK (Erk1/2) was determined by Western blotting using phospho-specific antisera. As shown in Figure 2, phosphorylation of both p42- and p44-MAPK isoforms was observed. Total p42-MAPK (Erk2) is shown in the upper panel as a loading control. Phosphorylated p38 or total p38 was not different between control and sildenafil treated mice.

Effect of ERK activation on iNOS/eNOS protein expression:

Heart samples were collected 24 hours after saline, sildenafil, PD98059 and followed by sildenafil, or PD98059 injection. Protein levels of iNOS/eNOS were determined by Western blotting. As shown in Figure 3, iNOS protein expression is increased with sildenafil as compared to the saline control group, and this increase is completely blocked by PD98059 pretreatment.

DISCUSSION

The expression of the iNOS gene is an important part of the body's response to stressors by modulating and regulating vascular smooth muscle tone and thus affecting function of several organ systems, including the cardiovascular system. The NO generated by iNOS

from its substrate L-arginine has beneficial effects (eg, antimicrobial, antiatherogenic, antiapoptotic), whereas the overproduction of induced NO has detrimental consequences (eg, direct cellular injury and proinflammatory response). Sildenafil pretreatment was shown to induce upregulation of iNOS, but the exact mechanism of this effect is not yet fully understood. Studies have suggested that the iNOS gene is regulated at multiple levels: transcriptional, posttranscriptional, and posttranslational. However, the role of the mitogen-activated protein kinase (MAPK) cascades in the control of iNOS expression has not been completely defined. In this study, we investigated whether the MAPK signaling pathway was involved in the regulation of iNOS expression and whether sildenafil influenced MAPK activity. In the present study, a single bolus injection of sildenafil of 0.71 mg/kg caused significant reduction in the infarct size 24 hrs later, when compared to the saline-treated controls. The selective inhibition of ERK by PD98059 completely abolished the protective effect of sildenafil whereas p38 MAPK inhibitor SB203580 failed to block the protection. These results are further supported by increased phosphorylation of p44/42 sildenafil treated hearts while absence of phosphorylated p38 in the same samples. No significant changes in the pre- or post-ischemic ventricular function were observed between the control and treated groups. Taken together, our results show that phosphorylation of ERK 1 and 2 mediates sildenafil induced delayed cardioprotection against global ischemia/reperfusion injury.

The role of p38 signaling in early preconditioning has been extensively investigated, although conflicting results have been obtained. However, relatively little is known about

the role of MAP kinases, in particular their cause and effect during delayed preconditioning. Ping et al [32] demonstrated that ischemic PC was associated with activation of p44 and p42 MAPKs in rabbit hearts although the role of these kinases in delayed protection in this model was not studied. Recently we showed that heat stress-induced delayed protection was mediated by MAP kinases. Dana et al [78] showed transient activation of adenosine A_1 receptor with 2-chloro-N^6-cyclopentyladenosine (CCPA) induced delayed preconditioning which was also accompanied by rise in the p38 activity. The delayed protection was abolished by p38 inhibitor, SB203580 in the mouse heart suggesting an essential role of p38 in protection. Recently, we showed that selective activation of p38 with anisomycin triggered delayed cardioprotection in the mouse heart. In this respect, it appears that sildenafil is the first drug which mediated delayed cardioprotection through phosphorylation of ERK 1/2.

In summary, we demonstrated that a significant ERK phosphorylation can be detected with 0.71 mg/kg i.p. administration of sildenafil, which is in a dose range that is clinically relevant. The results provide a possible mechanism to explain the potential infarct-limiting effect of sildenafil and suggest that ERK activation by this PDE-5 inhibitor may be involved in the subsequent induction of iNOS expression.

Figure Legends

Figure 1. Experimental protocol for Langfendorff isolated moude heart experiments.

Figure 2. Effect of sildenafil, SB203580, and PD98059 on ventricular functional recovery following global ischemia-reperfusion.

Figure 3.   Effect of sildenafil on myocardial infarct size: role of p38 MAPK and p44/42 MAPK.   Isolated perfused hearts were subjected to 20 min global ischemia and 30 min reperfusion in Langendorff mode. Values are means ± SE (n = 6 heart samples per group). Group III and V received SB-203580 (1 mg/kg, ip) or PD 98059 (1 mg/kg, ip) 30 min before sildenafil treatment.   Group IV and VI served as controls for SB203580 and PD98059,   respectively.   *P < 0.05 vs.   saline   control,   SB203580+saline, PD98059+sildenafil and PD98059+saline.

Figure 4. Western blots showing MAPK phosphorylation after treatment with sildenafil. Tissue   homogenates   were   immunoprecipitated   with   a   mouse   monoclonal antiphosphotyrosine   antibody   combined   with   protein   A/G   plus   agarose. Immunoprecipitates   were   subsequently   analyzed   by   Western   blots   using   a   mouse monoclonal antibody for a 1:100 dilution of either total and phosphorylated p38 MAPK or total and phosphorylated anti-ERK 1 and 2 rabbit polyclonal antibody.

Figure 5. Western blots showing cardiac expression of iNOS and eNOS proteins 24 hrs after sildenafil treatment with or without PD98059. Actin levels for each sample shows equal protein loading.
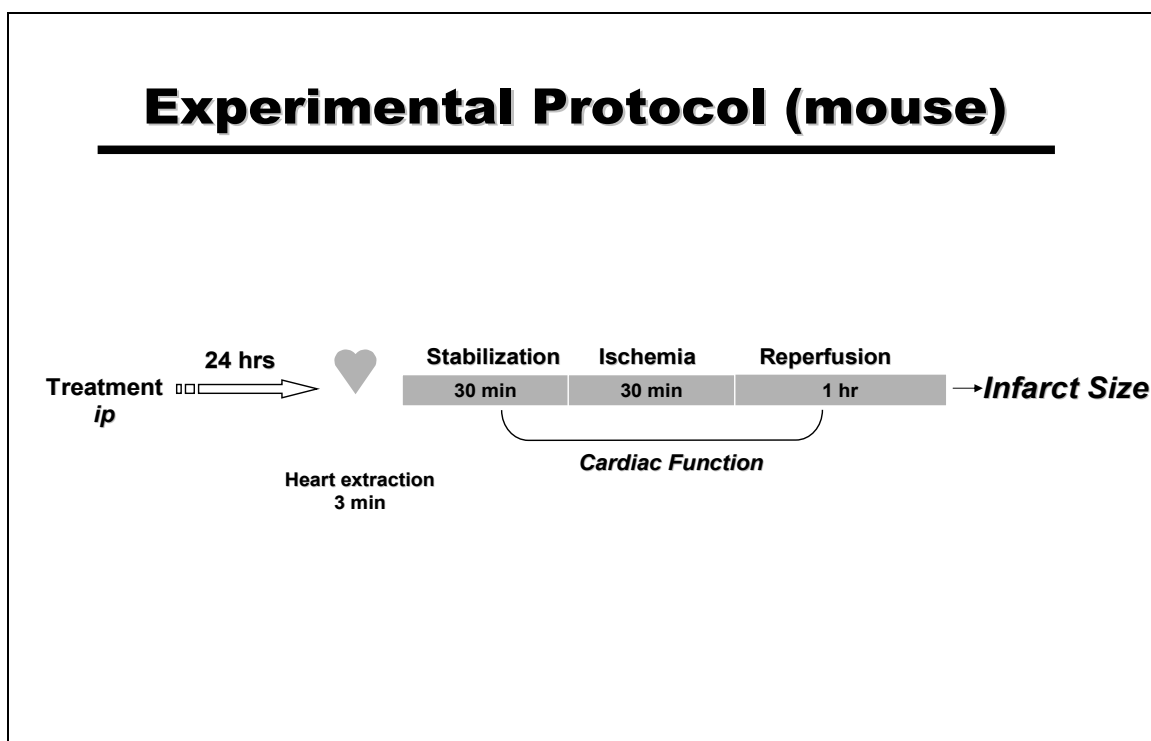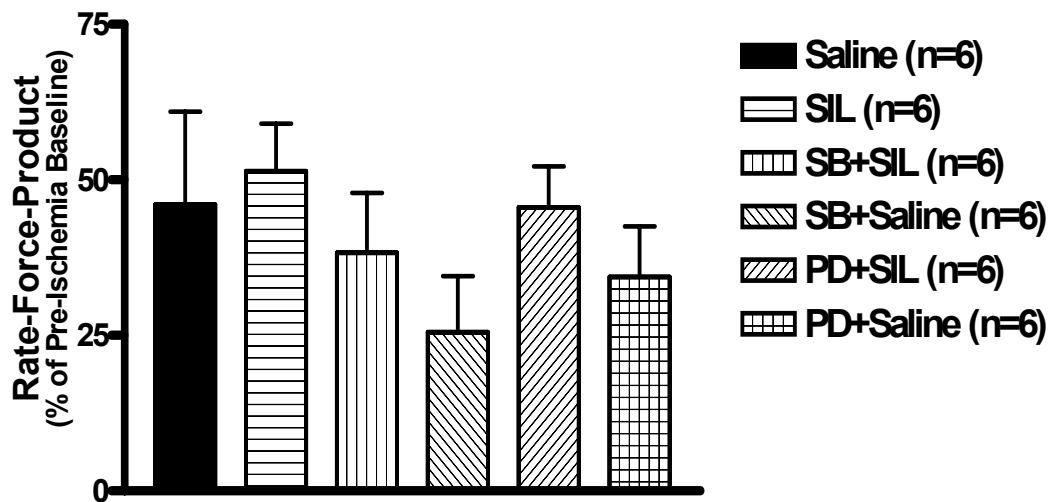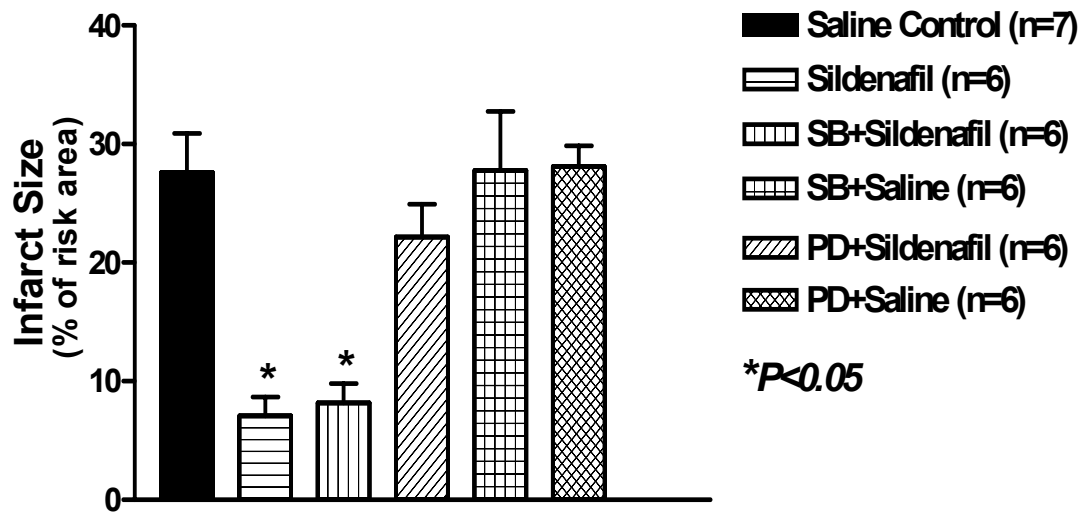
Figure 1

Figure 2

Figure 3

*P<0.05

Figure 4

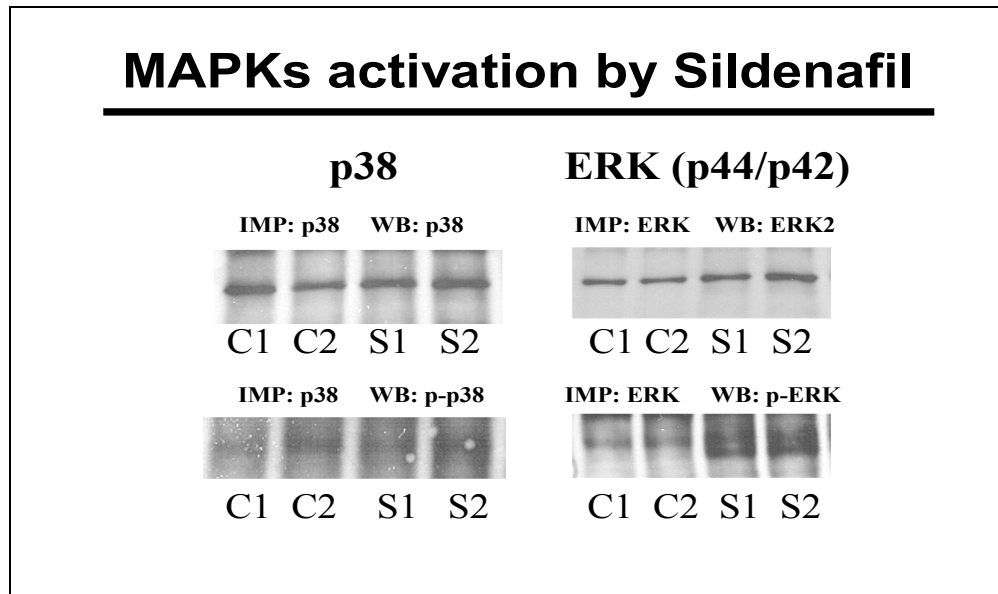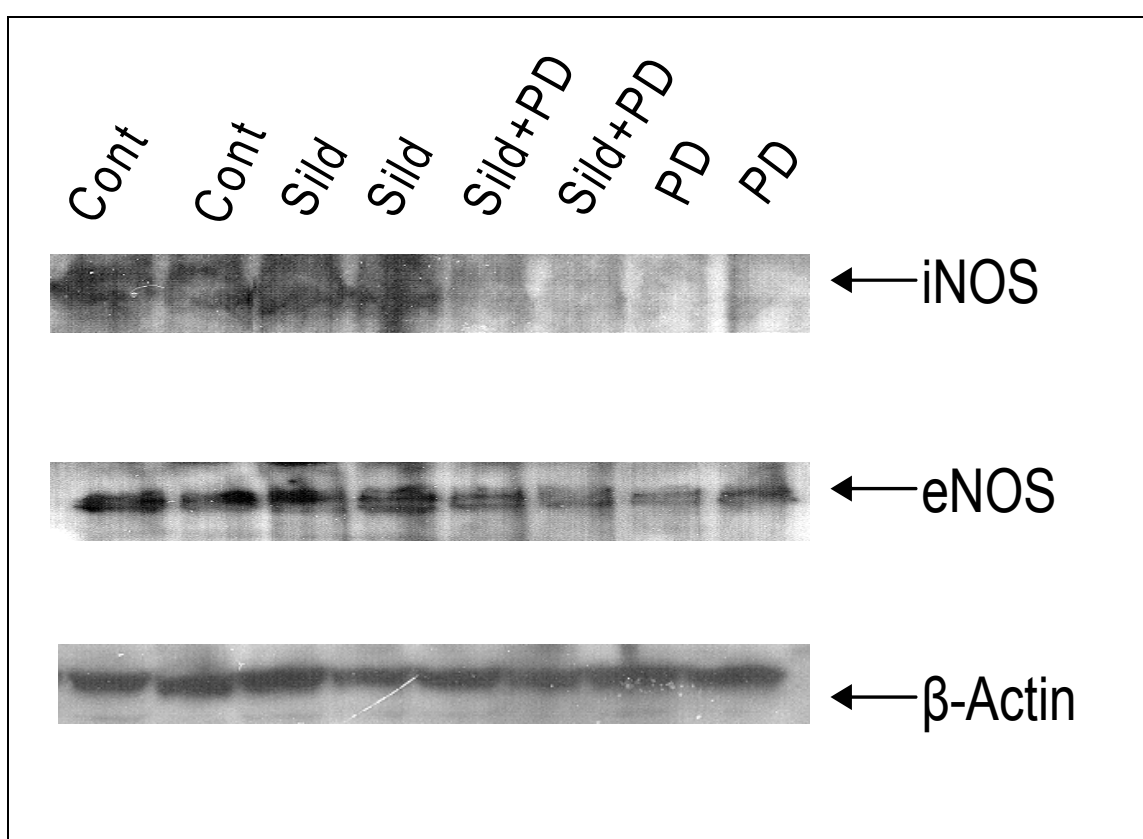

MAPKs activation by Sildenafil

C1 and C2 represent saline control
S1 and S2 represent sildenafil treatment

Figure 5

CHAPTER 7

Delayed Cardioprotection with Sildenafil in Mice: Role of Adenosine A1 Receptor and Its Genetic Deletion

Background: Since adenosine has been implicated to be a major trigger of schemic and pharmacological PC, we tested the hypothesis that A1 adenosine receptor (A1AR) activation plays a role in sildenafil-induced cardioprotective signaling.

Methods and Results: Adult male C57BL-wild type (WT) mice or their corresponding A1AR-knock out (KO) mice were pretreated (i.p.) with either sildenafil (0.71 mg/kg, equivalent to 50 mg dose for 70 kg patient) or volume-matched saline. The selective A1AR antagonist DPCPX (0.1 mg/kg, *i.p.*) was administered 30 min before sildenafil. The hearts were isolated 24 hours later and subjected to 30 min global ischemia and 1 hr of reperfusion in Langendorff mode. Infarct size was measured by computer morphometry of TTC stained sections. Post-ischemic myocardial infarct size (Mean±SEM; % of risk area) was reduced in C57BL-WT mice treated with sildenafil (5.6±0.9) versus saline group (27.3±2.1; P<0.05; n=6/each). Sildenafil failed to precondition the A1AR-KO hearts (31.6±1.9 vs. 32.3±1.5 with saline, p>0.05). Additionally, DPCPX treatment abolished the infarct limiting effect of sildenafil (27.3±3.2, p<0.05). DPCPX alone had no effect on infarct size as compared with the control group. No significant changes in left ventricular pressure and heart rate were observed in sildenafil-treated group.

Conclusion: The sildenafil-induced delayed protection against global ischemia/reperfusion is mediated by signaling pathway involving the interaction of endogenous adenosine with its A1 receptor.

INTRODUCTION

Adenosine receptor activation following PC protects the heart against reversible and irreversible ischemic injury in multiple species and preparations (97,98). As described in the previous section, our initial studies demonstrated the cardioprotective effect of sildenafil in several animal species. The hypothesis behind these studies was that the release of endogenous mediators such as adenosine, bradykinin or NO following sildenafil treatment. This in turn could potentially trigger signaling pathways leading to cardioprotection.

Since we previously demonstrated the importance of ERK activation and iNOS upregulation in cardioprotection with sildenafil, and since other studies have already demonstrated the link between A1AR and iNOS, we examined the possibility of A1AR contribution to the PC-like effect afforded by sildenafil. We hypothesized that sildenafil, through its hypotensive effects, would potentially lead to the release of adenosine from cells into the interstitial fluid, thus making adenosine available to interact with its receptors. This mimics, to a certain extent, the release of small amounts of adenosine observed during brief episodes of ischemia that leads to PC against subsequent prolonged ischemia. In the present study, we took advantage of adenosine A1 receptor knock-out

mice and selective A1 receptor antagonist DPCPX to elucidate the role of A1AR in inducing delayed cardioprotection in mice. The goals of the study are as follows:

1) to determine whether sildenafil could confer cardioprotective effects in the C57BL-WT mice similar to ICR outbred mice shown previously; 2) to show if sildenafil confers delayed cardioprotection in A1AR-KO mice and following inhibition of the receptor in WT mice with the selective A1AR antagonist DPCPX; and 3) to demonstrate that sildenafil induces protection against simulated ischemia/reoxygenation in mouse cardiomyocytes derived from WT and A1AR-KO mice.

Materials and Methods

Animals

Adult male C57BL mice were supplied by The Jackson Laboratory (Bar Harbor, ME. The body weight for these animals ranged between 27.1 and 32.7 g. A1AR-KO mice were provided by The National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20592; PI Dr. Jurgen Schnermann.

Langendorff Isolated Perfused Heart Preparation

The methodology of the Langendorff isolated perfused heart has been described in previous chapters. The only difference in the current study in the use of left ventricular developed pressure as opposed to ventricular contractile function. To obtain the developed pressure, a left atrial incision was made to expose the mitral annulas through which a water-filled latex balloon was passed into the left ventricle (LV). The balloon was attached via polyethylene tubing to a Gould pressure transducer that was connected to a PowerLab Acquisition System (ADInstruments 8SP, Australia). The balloon was inflated to adjust the LV enddiastolic pressure (LVEDP) to _10 mmHg. Myocardial ischemic damage was measured using multiple, independent end points of tissue injury. These included infarct size, LV developed pressure (LVDP), LVEDP, rate-pressure product (RPP), heart rate, and coronary flow by timed collection of the perfusate. LVDP was calculated by subtracting LVEDP from the peak systolic pressure. RPP, an index of cardiac work, was calculated by multiplying LVDP with heart rate. The hearts were not paced.

Study Protocol

Mice were randomly assigned into one of the following groups:

Group I: (saline control, n=6): WT mice received 0.9 % saline, *i.p.*;

Group II: (saline control, n=6): A1AR-KO mice received 0.9% saline, *i.p.*;

Group III: (Sildenafil, n=6): Wt mice; pure sildenafil powder provided by Pfizer, Inc.was dissolved in saline. This preparation was given as 0.71 mg/kg *i.p.* bolus, approximating, on a mg/kg basis, the clinical dose of 50 mg administered to a 70 kg patient;

Group IV: (Sildenafil, n=6): A1AR-KO mice received 0.71 mg/kg sildenafil as in Group III;

Group V: (DPCPX+sildenafil n=6): WT mice; treatment with DPCPX (0.1mg/kg) 30 min prior to sildenafil treatment as in Group III

Group VI: (DPCPX+saline n=6): WT mice; treatment with DPCPX (0.1mg/kg) 30 min prior to saline treatment as in Group I.

Twenty-four hours later, the hearts were isolated and following a 30 min stabilization period, the hearts were subjected to 30 min no-flow normothermic global ischemia and 1 hr reperfusion.

Another subset of mice was used for adult cardiomyocyte isolation as described later in this section. The isolated cardiomyocytes were subjected to hypoxia/reoxygenation to simulate ischemia/reperfusion in the intact heart. Different study groups were randomly assigned in order to assess the role of sildenafil in cardioprotection *in vitro* as well as evaluating the role of the A1AR in the sildenafil induced protection.

Isolation of adult mouse ventricular cardiomyocytes: Adult male C57BL-WT (The Jackson Laboratory, Bar Harbor, ME) or A1AR-KO mice (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20592; PI Dr. Jurgen Schnermann) were used in this study. The animal experimental protocols were approved by the Institutional Animal Care and Use committee of Virginia Commonwealth University. The ventricular cardiomyocytes were isolated using an enzymatic technique modified from the previously reported method (95,96). In brief, the animal was anesthetized with pentobarbital sodium (100 mg/kg, i.p.) and heart was quickly removed. Within 3 min, the aortic opening was cannulated onto a Langendorff perfusion system and heart was retrogradely perfused (37^oC) at a constant pressure of 55 mmHg for ~5 min with a Ca^{2+}-free bicarbonate-based buffer containing (in mM): 120 NaCl, 5.4 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 5.6 glucose, 20 $NaHCO_3$, 10 2,3-butanedione monoxime, and 5 taurine, which was continuously bubbled with $95\%O_2 + 5\%CO_2$. The enzymatic digestion was commenced by adding collagenase type II (Worthington, 0.5 mg/mL each) and protease type XIV (0.02 mg/mL) to the perfusion buffer and continued for ~15 min. 50 μM Ca^{2+} was then added in to the enzyme solution for perfusing the heart for another 10-15 min.

The digested ventricular tissue was cut into chunks and gently aspirated with a transfer pipette for facilitating the cell dissociation. The cell pellet was resuspended for a 3-step Ca^{2+} restoration procedure (*i.e.* 125, 250, 500 µM Ca^{2+}). The freshly isolated cardiomyocytes were then suspended in minimal essential medium (Sigma catalogue# 6 M1018, pH 7.35-7.45) containing 1.2 mM Ca^{2+}, 12 mM $NaHCO_3$, 2.5% fetal bovine serum and 1% penicillin-streptomycin. The cells were then plated onto the 35 mm cell culture dishes, which were pre-coated with 20 µg/mL mouse laminin in PBS + 1% penicillin-streptomycin for 1 hour. The cardiomyocytes were cultured in the presence of 5% CO_2 for 1 hour in a humidified incubator at 37^oC, which allowed cardiomyocytes to attach to the dish surface prior to the experimental protocol.

Experimental Protocol

The cultured cardiomyocytes were incubated under 37^oC and 5% CO_2, for 1 hour with or without 1 µM sildenafil citrate powder, dissolved in distilled water. The drug solution was filtered (0.45 µm pore size) before adding into cell medium. Cardiomyocytes were subjected to SI for 40 minutes by replacing the cell medium with an "ischemia buffer" which contained (in mM): 118 NaCl, 24 $NaHCO_3$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$-$2H_2O$, 1.2 $MgCl_2$, 20 sodium lactate, 16 KCl, 10 2-deoxyglucose (pH adjusted to 6.2). In addition, the cells were incubated under hypoxic conditions at 37^oC during the entire SI period by adjusting the tri-gas incubator to 1-2% O_2 and 5% CO_2. RO was accomplished by replacing

the ischemic buffer with normal medium under normoxic conditions. Assessment of cell necrosis and apoptosis was performed at 18 hours of RO.

Evaluation of Cell Viability: Cell viability was assessed by trypan blue exclusion assay. At the end of protocol, 20 µL of 0.4% trypan blue (Sigma-Aldrich) was added into the culture dish. After ~5 min of equilibration, the cells were counted under microscope.

Statistics: All measurements are expressed as means ± SE. The data were analyzed by either unpaired *t*-test or one-way ANOVA. If a significant value of *F* was obtained in ANOVA, the Student-Newman-Keuls post hoc test was further used for pairwise comparisons. Paired *t*-test was used to compare any pair of pre- and post-treatment values for the same parameter. $P < 0.05$ was considered significant.

RESULTS

Cardiac Hemodynamics and Contractile Function:

There was no significant difference in the basal functional parameters (*i.e.* developed LV pressure, rate-pressure product, and EDP) between the groups after 20 min stabilization. During post-ischemia also, there was a significant increase in the rate pressure product (Figure 1) only in the sildenafil-treated group. Post-ischemic coronary flow was not significantly different among the groups.

Myocardial Infarction:

Post-ischemic myocardial infarct size (Mean±SEM; % of risk area) was reduced in wild type mice treated with sildenafil (5.6±0.9) versus saline group (27.3±2.1; P<0.05; n=6/each) (Figure 2). On the other hand, sildenafil did not offer any protection in the A1AR-KO mice (31.6±1.9 vs. 32.3±1.5 with saline, p>0.05). Moreover, the selective A1AR antagonist DPCPX completely blocked the infarct-limiting effect of sildenafil in the wild type mice (27.3±3.2, p<0.05). There was no significant difference in the infarct size between the controls and the animals treated with DPCPX or DMSO, the solvent for DPCPX.

Effect of Sildenafil on Cardiomyocyte Necrosis

Our method for cell preparations yielded a relatively high percentage of the cardiomyocytes with rod shape morphology, which was similar to previously reported studies (95,96).

After 40 min of SI and 18 hours of RO, the trypan blue positive cardiomyocytes reached 53±2.8%. However, prior treatment with sildenafil reduced the trypan blue positive cells as compared with untreated SI-RO group (24.5±2.6, p<0.05, n=3; Fig 3). The A1AR selective

antagonist, DPCPX, abolished the protective effect of sildenafil as shown by an increase in the trypan blue positive cells which was not different from control (50.8 ± 3.0). Interestingly, in the cardiomyocytes obtained from A1AR-KO mice, sildenafil failed to protect the myocytes against necrosis caused by H/R as compared with control (46.7 ± 2.3 vs 51.9 ± 4.4). DPCPX or its solvent DMSO alone had no effect on trypan blue positive cell count.

Discussion

Since sildenafil causes a hypotensive response, it is possible that adenosine would be released and consequently interacts with its receptors potentially leading to a preconditioning-like effect. Accordingly, the previously reported cardioprotective effect of sildenafil could be mediated by A1AR. In the present study, we used a reltively selective approach by employing mice showing genetic deletion of the A1AR and we investigated the role of the A1AR in the sildenafil-induced preconditioning-like effect. As our recent results show, sildenafil did in fact confer powerful cardioprotection against I/R injury in an isolated mouse heart model of I/R. This protection was abolished by both the selective A1AR antagonist DPCPX and genetic deletion of A1AR. Furthermore, in the isolated adult cardiomyocyte derived from wild-type mice, the trypan blue positive necrotic cells were significantly lower in the sildenafil-treated group as compared with the saline treated control myocytes. Also, sildenafil-induced protection was abolished by A1AR antagonist, DPCPX and cells obtained from A1AR-KO mouse cardiomyocytes treated with sildenafil.

Adenosine receptor subtypes, including the A1 and A2a receptors, have been reported to couple to the ERK pathway in both cardiac and noncardiac tissues. It has also been reported that the beneficial effects of adenosine A2a receptor activation during reperfusion appear to be due to ERK activation (107). However, thusfar, there have been no studies examining whether adenosine receptor PC is mediated via ERK. Although the results of a significant number of studies suggest that ERK activation during ischemia-reperfusion is beneficial, there are additional reports suggesting otherwise. The discrepancies in the exact role of ERK activation in myocardial ischemia-reperfusion may be due to the specific stimulus and the duration of ERK activation. Despite the key role for mitochondria in mediating the beneficial effects of PC, as well as contributing to reperfusion oxidative stress, and a report that ERK isoforms are present in murine heart mitochondrial fractions (108), there have been no reports examining mitochondrial ERK activation during PC or myocardial ischemia/reperfusion.

Our present results raise a very interesting topic as to how sildenafil could afford cardiomyocyte protection against H/R in the absence of the hypotensive effect. If the mild drop in blood pressure due to cGMP accumulation is what caused adenosine release *in vivo* as we initially hypothesized, then there probably is some other mechanism through which sildenafil is effective against necrosis from H/R *in vitro*. It is possible that cGMP formed after treatment with sildenafil somehow affects the A1AR sensitivity and thus allows more adenosine to re-enter the cell after its release during hypoxia and thus generating a

preconditioning-like effect. More research is needed to address this interesting finding and further investigate the exact cause-and-effect relationship between sildenafil and the A1AR.

In conclusion, we have demonstrated in the current study that genetic deletion of A1AR abolishes the cardioprotective effects of ischemic preconditioning as well as pharmacological preconditioning with the PDE-5 inhibitor sildenafil. Our findings do in fact corroborate a key role of A1AR activation in the subcellular mechanisms of myocardial protection against I/R injury.

Figure Legends

Figure 1. Experimental protocol showing the various groups.

Figure 2. Effect of sildenafil, DPCPX, and genetic deletion of A1AR on ventricular functional recovery following global ischemia-reperfusion.

Figure 3. *TOP:* Representative heart sections showing infarction (white) with 10% TTC staining. More viable tissue (red) is prominent in the sildenafil-treated WT mice as compared to the A1AR-KO mice and DPCPX-treated mice.

BOTTOM: Effect of sildenafil on myocardial infarct size: role of A1AR. Isolated perfused hearts were subjected to 30 min global ischemia and 1hr reperfusion in Langendorff mode. Values are means ± SE ($n = 6$ heart samples per group). Group III (WT) and IV (A1AR-KO) received sildenafil (0.71 mg/kg, ip). Group V received DPCPX (0.1 mg/kg) 30 min before sildenafil treatment. Group (I, II) and (VI) served as controls for sildenafil and DPCPX, respectively. *$P < 0.05$ vs. sildenafil+WT.

Figure 4. Necrotic index using trypan blue in adult ventricular cardiomyocytes obtained from wild type and A1AR-knock out mice.

Figure 5. Digital photo showing an adult mouse heart being perfused in Langendorff mode.

Figure 1

Experimental Protocol

Saline+WT
Saline+KO
SIL+WT
(0.7 mg/kg, ip)
SIL+KO
(0.7 mg/kg, ip)
DPCPX
(0.1mg/kg, ip)
+SIL
(0.7mg/kg, ip)
DPCPX
(1mg/kg, ip)
+Saline

Stabilization	Ischemia	Reperfusion
30 min	30 min	60 min

Cardiac Function

Infarct Size
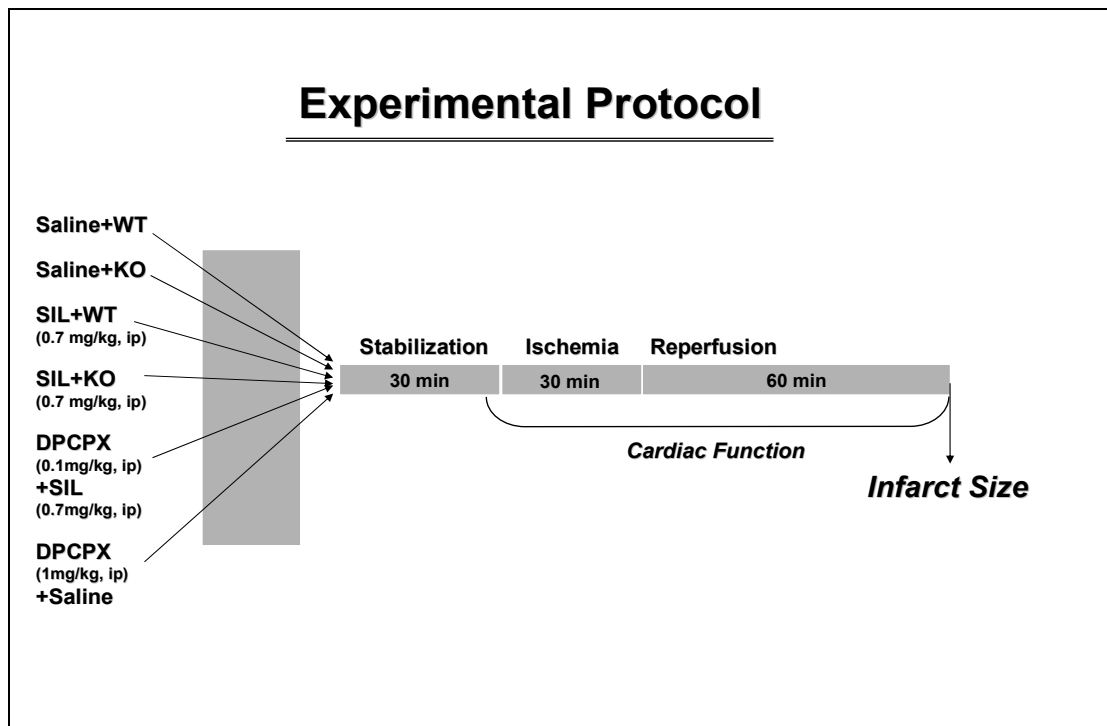
Figure 2

Figure 3

Sildenafil+C57BL-WT
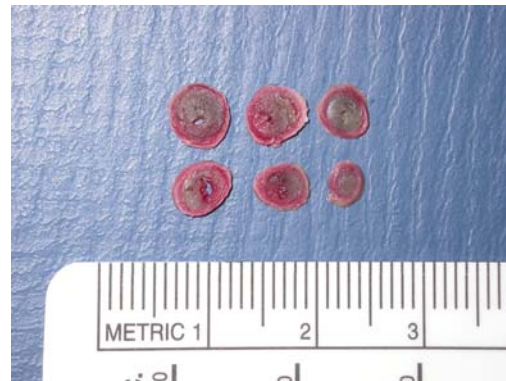
DPCPX+Sildenafil+C57BL-WT

Sildenafil+A1AR-KO

Legend:
- WT+Saline (n=6)
- KO+Saline (n=6)
- WT+SIL (n=6)
- KO+SIL (n=6)
- WT+DPCPX+SIL (n=6)
- WT+DPCPX+Saline (n=6)

*$P<0.05$
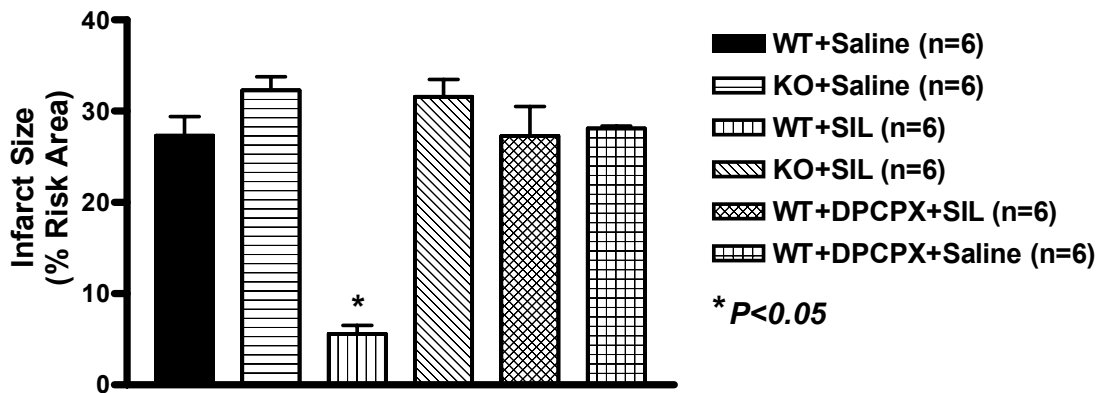
Figure 4

Figure 5

Chapter 8

General Discussion

Phosphodiesterase type-5 (PDE-5) inhibitors

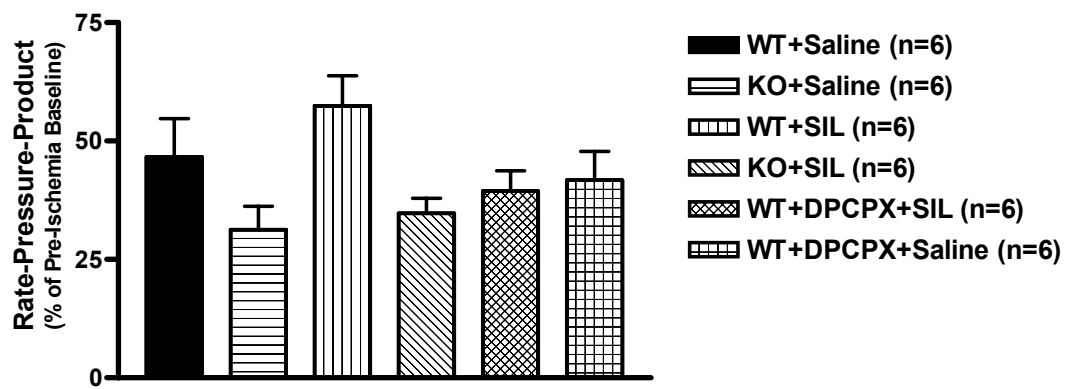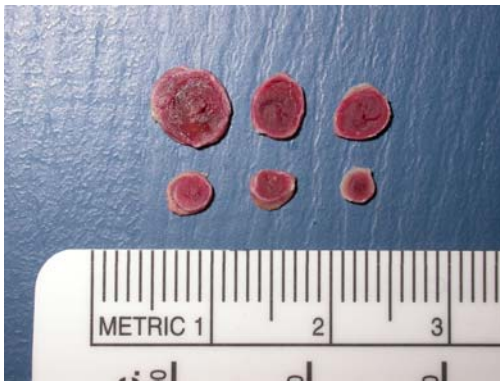PDE-5 inhibitors are a new class of vasoactive drugs that have been developed for treatment of ED in men. Their main mechanism of action is through the active inhibition of PDE-5 enzyme thus allowing accumulation of cGMP resulting in smooth muscle relaxation in the penis. So far, there are 11 known families of PDEs shown to exist in mammalian tissues. Even though PDEs are expressed in all tissues, the distribution of the various isoforms may differ among various tissues and cell types. These enzymes play a key role in modulating diverse physiological processes.

Sildenafil citrate (Viagra^{TM}) is the first oral agent approved for treatment of ED in men. Its chemical structure is similar to that of cGMP and therefore inhibits PDE-5 by binding to the cGMP-catalytic sites (Corbin and Francis, 2002), resulting in the accumulation of cGMP in the erectile tissue.
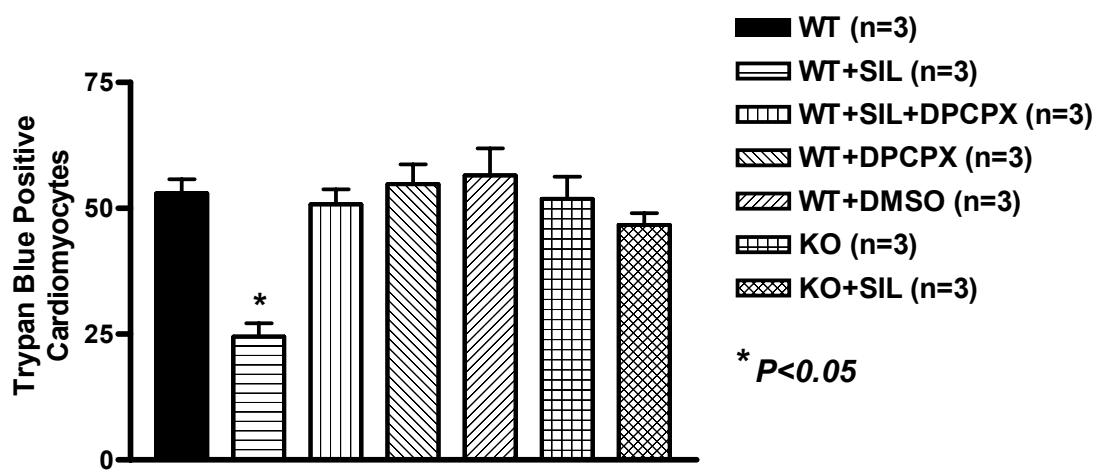
Cardioprotection with sildenafil against I/R injury

For a little over three years now, sildenafil has been investigated as a candidate for pharmacological PC against I/R injury in animal models. Due to its potent inhibition of

PDE-5, we hypothesized that such vasodilatory effect caused by cGMP in the vascular smooth muscle bed may potentially result in PC-like effect through affecting various pathways implicated in PC. This pioneering work was initiated in our lab and was first reported in 2002 (chapter 2). More research was conducted to elucidate the mechanism through which sildenafil could confer powerful acute and delayed PC-like effects.

First, our results from the first study indicated the importance of mitoKATP channel opening in the cardioprotection afforded by sildenafil when administered at a clinically relevant dose; 0.71 mg/kg that was based on a 50 mg sildenafil tablet given to a 70 kg patient as previously discussed (chapters 2, 3,4,5,6 and 7). This was shown by the ability of 5-HD, a selective mitoK$_{ATP}$ channel blocker, to completely abolish the protective effect of sildenafil in our well-established rabbit model. Several studies have now conclusively demonstrated that opening mitoK$_{ATP}$ channels plays an important role in ischemic as well as pharmacological preconditioning in the heart. K$_{ATP}$ channels were first identified by cardiovascular physiology studies as causing vascular smooth muscle relaxation in either large or small arteries or as having a negative inotropic effect on cardiac myocytes. In addition, activation of these channels seems to occur as a response to decreased intracellular ATP levels, which is normally achieved within a few minutes after the onset of ischemia. Because K$_{ATP}$ channels act as an inward rectifier when activated, these effects could be caused by an increase of the depolarization threshold that reduces excitation of either vascular smooth muscle or cardiac myocytes followed by vasodilation and shortening of the action potential duration, respectively, which finally lead to intracellular

Ca^{2+} unloading and, as a result, reduced metabolic demand. Since these phenomena resemble the acute cardiac responses and cardioprotection afforded by ischemic PC, it has been postulated that $mitoK_{ATP}$ channels might be one of the critical effectors of both ischemic and pharmacological preconditioning.

In the rabbit studies (chapters 2,4 and 5), sildenafil caused an intense effect on the hemodynamics. However, this profound drop in blood pressure bounced back to baseline value within 5 minutes post treatment with sildenafil. Despite these pressure variations, heart rate was not considerably affected by sildenafil administration.

In chapter 3, we further investigate the mechanism(s) through which sildenafil could cause a reduction in infarct size following I/R in the rabbit heart. Since PKC is a key player in PC, and translocation of some of its various isoforms is believed to alter several subcellular pathways including $mitoK_{ATP}$ channel opening, we sought to investigate the role of PKC in the sildenafil induced cardioprotection. The PKC inhibitor, chelerythrine, blocked the PC-like effect of sildenafil in rabbits (chapter 3). Furthermore, activation, i.e., translocation of PKC-α, -θ and -δ isoforms from cytosol to the membrane fraction after treatment with sildenafil was demonstrated by Western blot analysis. On the other hand, no change in PKC-β and -ϵ isoforms was observed.

In chapter 4, we examined the cardioprotective effects of sildenafil in infant rabbits. Each year, more than 25,000 children endure corrective heart surgery for congenital cardiovascular disease. These infants are at risk for myocardial ischemia during cardiopulmonary bypass, circulatory arrest or low flow states which are inevitable components of the procedure (Rafiee et al., 2003). Despite the data supporting the higher

flexibility of the infant myocardium to ischemic injury as compared to adults, the infant heart responds quite differently to cardiovasular drugs, stress, and changes in hemodynamics (Lewin et al., 1998; Wernovsky et al., 2001). The procedures currently used in infants are not without limitations, such as poor functional recovery and even mortality. For this reason, there is a great necessity for developing pharmacological agents to be used as adjunct therapy along with surgical intervention in order to minimize, if not eradicate, the side effects shown so far. In this respect, we examined the effect of sildenafil on infarct size reduction as well as functional recovery following I/R in 8-week old infant rabbits (chapter 5).

In addition to monitoring the hemodynamic changes as an indication of LV function , we assessed the left ventricular cardiac output (LVCO) and aortic velocity time integral (VTI) using trans-esophageal echocardiography (TEE). Our results were analogous to those obtained from our adult rabbit experiments. We found that sildenafil could confer cardioprotection against I/R in the infant rabbit heart. Both the control and sildenafil-treated groups had similar LVCO and aortic VTI at baseline. However, as the TEE results indicated, only the sildenafil-treated group showed enhanced aortic VTI and LVCO 30 min following ischemia. A descent in both values was reported at 3 hr of reperfusion in all groups, but it was not statistically significant in the sildenafil-treated group.

Other studies, also from our lab, were conducted using isolated mouse hearts perfused in Langendorff mode as previously described (chapters 3,6 and 7). Our goal was to further elucidate the mechanism of action of sildenafil in cardioprotection.

NO has been long implicated as an essential trigger and mediator of delayed PC. It has been also shown to be involved in physiological as well as pharmacologival PC. NO is synthesized by 3 isoforms of NO synthases (NOS), specifically, nNOS, iNOS and eNOS. Since it has been shown that NO derived from iNOS is the mediator of delayed PC, we tested the hypothesis that sildenafil treatment has an up-regulatory effect on iNOS leading to increased NO production. Our results showed a significant reduction in infarct size following global I/R in the sildenafil-treated mice as compared to the corresponding controls 24 hr post-treatment. Also, the infarct-sparing effect of sildenafil was abolished by the selective iNOS inhibitor, 1400W, which had no effect on infarct size when administered alone. Sildenafil treatment did not alter pre- or post-ischemic coronary flow, indicating that its cardioprotective effect may be independent of its vasodilatory effect in the delayed phase. RT-PCR showed a transient increase in the levels of both eNOS and iNOS, peaking at 45 minutes (eNOS) and 2 hours (iNOS) after sildenafil treatment and returning to baseline levels several hours later (chapter 3), and this increase; however, was more profound for iNOS mRNA. Moreover, Western blot analysis revealed a significant increase in cardiac expression of iNOS and eNOS proteins 24 hours after sildenafil treatment. These findings confirm that iNOS mediates the sildenafil-induced delayed PC in mice.

The mitogen-activated protein kinase (MAPK) family has been associated with ischemic PC. The results; however, have been divisive. Activation of p38 by anisomycin mimicked PC and delayed cardioprotection in the isolated perfused hearts (Zhao et al., 2001). In the MAPK family, the p42/p44-MAPK or ERK cascade appears to mediate cell growth and

survival signals in many cell types. Sustained activation of p42/p44-MAPK during simulated 'reperfusion' following sublethal simulated ischemia mediates PC in cardiomyocytes independent of transient activation of p38 MAPK (Punn et al., 2000). Our preliminary results (chapter 6) have shown that selective inhibition of ERK by PD98059 completely abolished the delayed protective effect of sildenafil whereas p38 MAPK inhibitor SB203580 failed to block the protection. These results were further supported by increased phosphorylation of p44/42 but not p38 in sildenafil treated hearts. Furthermore, PD98059 treatment prior to sildenafil blocked the increase of iNOS protein 24 hr later, as shown by Western blot analysis, indicating that sildenafil upregulates iNOS through ERK phosphorylation (chapter 6).

Most recently, we tested the hypothesis that the adenosine A1 receptor plays a role in PC with sildenafil. The reason behind this study was that role of A1AR in PC has been extensively studied, but the results have been inconclusive due to the limitations of the A1AR antagonists. As a result of myocardial ischemia, there is an immediate depletion in the ATP that is available for the cardiac cells and as a consequence adenosine is released to interact with its receptors. In this respect, it was believed that brief episodes of I/R would cause the release of adenosine and allow the interaction with its receptors to "precondition" the myocardium against future ischemic insults. Moreover, intracoronary infusion of adenosine in the isolated rabbit heart or CCPA in the isolated mouse heart prior to sustained I/R demonstrated a marked decrease in infarct size when compared to control animals. Due to the recent availability of A1AR-KO mice, the role of this receptor could be further examined. For this reason, we tested the role of A1AR in the infarct-limiting

effect of sildenafil. Our results demonstrated that the sildenafil-induced delayed protection was abolished in the intact heart as well as adult myocytes derived from adenosine A1 receptor knock-out mice suggesting an essential role of A1 receptor in protection.

Taken together, these studies suggest that sildenafil is a powerful tool to reduce I/R injury in the animal models studied thus far. The ongoing research effort from our lab provided a deeper perspective for understanding the signaling mechanisms of cardioprotection induced by sildenafil, apart from its well known function in causing cGMP accumulation in the vascular smooth muscle bed. All these findings could be summarized as follows: 1) increased expression of nitric oxide synthases, 2) activation of kinases (such as PKC and ERK), 3) opening of mitochondrial K_{ATP} channels, and 4) activation of A1AR.

Conclusion and Future Directions

Since cardiovascular disease remains the number one cause of morbidity and mortality in the western world, it is necessary to develop novel pharmacological agents to be used as adjunct therapy in addition to the widely practiced surgical intervention. These invasive interventions are not without limitations and risks that might further damage an ischemic heart. Thus far, all the pharmacological agents that showed excellent cardioprotection in the animal models failed to do so to the same extent in human subjects. Since sildenafil is FDA approved, the overall safety margin of this drug has been well established. It is possible, due to its wide mechanism of action on the cellular and molecular level, that sildenafil may potentially succeed in conferring a PC-like effect

clinically in a similar fashion to the experimental setting. Therefore, vigilant clinical studies are needed to examine the role of sildenafil in PC in patients with ischemic heart disease. Preliminary experimental data from our lab (data not shown) have shown that another PDE-5 inhibitor, Vardenafil, exerts similar PC-like cardioprotective effects in the rabbit heart. Future clinical research is required to determine the safety and efficacy of sildenafil and other FDA-approved PDE-5 inhibitors in management of patients with coronary artery disease and to launch a novel cardioprotective strategy against I/R injury.

Figure 1
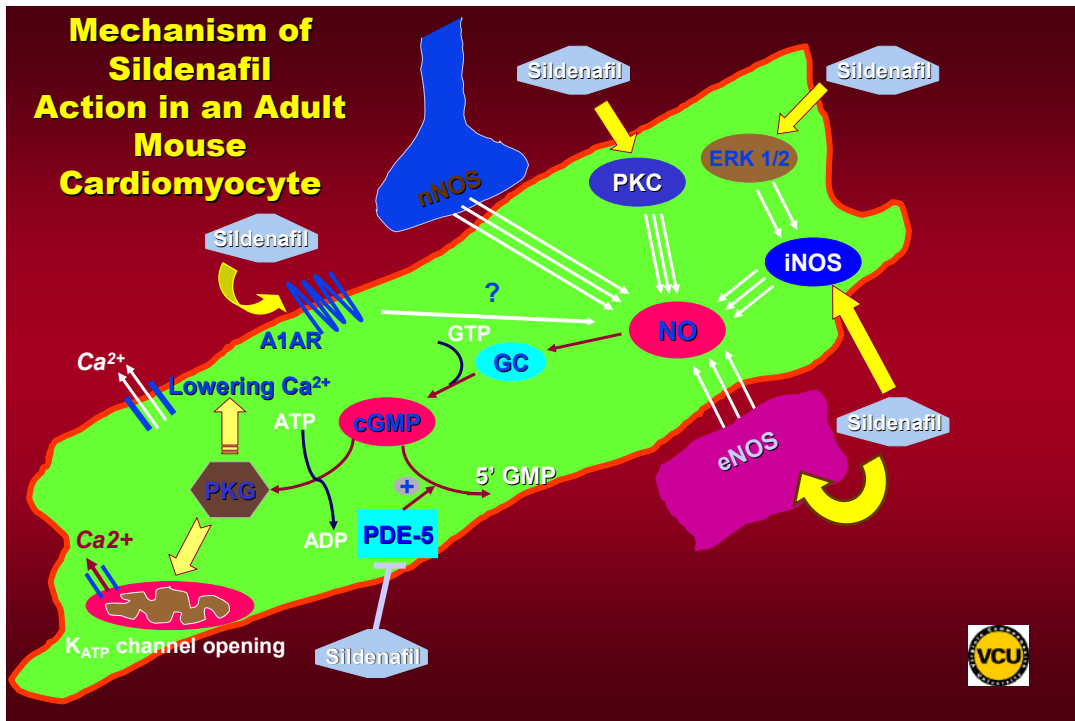
Mechanism of Sildenafil action in the mouse cardiomyocyte

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<u>VITA</u>

1. Born: August 11, 1975, Beirut, Lebanon

2. CITIZINSHIP: Lebanon/Green Card Holder; USA

3. EDUCATION

Degree	Subject	Institution	Years
B.S.	Biology	American University of Beirut, Beirut, Lebanon	1993-1996
Ph.D.	Physiology	Virginia Commonwealth University School of Medicine Richmond, VA	1999-2005

GRADUATE EDUCATION/RESEARCH

Virginia Commonwealth University School of Medicine, Department of Medicine and Physiology
Doctor of Philosophy in Physiology**, May 21, 2005**
Concentration: **Molecular and Cellular Cardiology**
Dissertation Title: **Novel Strategies in Cardioprotection against Ischemia/Reperfusion Injury**
Research Mentor: **Rakesh C. Kukreja, PhD**

MILITARY SERVICE RECORD 1997-1998

Lieutenant, Lebanese Army Medical Brigade

HONORS & AWARDS

Faculty Promotion Committee 2004-2005

Appointed by the Chairman of Physiology, Dr. Margaret Biber

Virginia Commonwealth School of Medicine
Medical College of Virginia
Department of Physiology

Assist in evaluation of faculty for promotion

SCHOLARLY, RESEARCH OR TEACHING EXPERIENCE

Graduate Students Trained

1. Marc Caligtan, M.S. student, Department of Physiology, 2003-2004

2. Sung Ko, M.S. student, Department of Physiology, 2003-2004

3. Christopher Thomas, M.S. student, Department of Physiology, 2004-present

4. Vladimir Daoud, M.S. student, Department of Physiology, 2004-present

Cardiology Fellow Trainees in basic research

1. Venkata R. Emani, M.D., Internal Medicine/Cardiology, 1999-2000

2. John W. Hawkins, M.D., Internal Medicine/Cardiology, 2001-2002

3. Chartchai Kositprapa, M.D., Internal Medicine/Cardiology, 2001-2002

4. Yvonne A. Bremer , M.D., Internal Medicine/Pediatric Cardiology, 2002-2003

5. Eric Chou, M.D., Internal Medicine/Cardiology, 2002-2003

6. Juan E. Weksler, M.D., Internal Medicine/Cardiology, 2002-2003

7. Mike J. Wittkamp, M.D., Internal Medicine/Cardiology, 2003-2004

8. Vijay Marwaha , M.D., Internal Medicine/Cardiology, 2003-2004

9. Stephen Zouzoulas, M.D., Internal Medicine/Cardiology, 2004-present

Major Teaching Assignments

1. PHIS 206: Human Physiology Lab, Medical College of Virginia Campus, VCU, Richmond VA

Invited Lectures

1. 50th Annual Virginia Thoracic Society Conference and Research Symposium, Richmond, VA, October 3, 2003

2. Pulmonary and Critical Care Medicine Conference Virginia Commonwealth University Medical Center Richmond, VA, March 7, 2005

National Scientific Conference Presentations

1. American Heart Association, Chicago, IL, November 18, 2002

Professional Societies

1. American Heart Association--Basic Cardiovascular Science Council

2. American Physiological Society

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In preparation:

13. Emani VR, Ockaili RA, **Salloum F** and Kukreja RC. Peroxynitrite Directly Triggers Early and Delayed Preconditioning in vivo via Opening of Mitochondrial K_{ATP} channels in the Rabbit Heart. In preparation

14. Ockaili RA, **Salloum F**, Murthy KS, Kukreja RC Role of Cyclic Guanosine Monophosphate (cGMP) in Sildenafil-induced cardioprotection. In preparation

15. **Salloum F,** Das A, Xi L, Ockaili RA, Yin C and Kukreja RC. Viagra (Sildenafil) induces delayed preconditioning against myocardial infarction by phosphorylation of ERK in mouse heart. In preparation

16. **Salloum F,** Ockaili RA, Chou E, Bremer Y and Kukreja RC. Sildenafil but not Nitroglycerine Administered at the Onset of Reperfusion Protects the Myocardium through Direct Opening of Mitochondrial KATP Channel: Lack of the Role of Nitric Oxide. In preparation

17. **Salloum F,** Ockaili RA, Chou E, and Kukreja RC. Myocardial Preconditioning with Sildenafil: The Effect of Red Wine Consumption. In preparation

18. Chou E, **Salloum F,** Ockaili RA, Bremer YA and Kukreja RC. Atorvastatin Triggers Acute and Delayed Preconditioning In-vivo: The Role of Nitric Oxide and K_{ATP} Channels. In preparation

Abstracts

1. Kositprapa C, Ockaili RA, **Salloum F**, Kukreja RC. Role of bradykinin B-2 receptor in late preconditioning: Evidence that opening of mitochondrial K-ATP channel functions as a trigger in delayed cardioprotection. *Circulation* 104 (17): 1089 Suppl. S OCT 23 2001

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11. Kukreja RC, Yin C, **Salloum F**, Xi L. Sildenafil (Viagra) induces delayed preconditioning through iNOS-dependent pathway in mouse heart. *European Heart Journal* 24: 476-476 Suppl. S AUG SEP 2003

12. Xi L, Taher M, **Salloum F** and Kukreja RC.   Cobalt Chloride Induces Delayed Preconditioning in Mouse Heart: Role of Hypoxia-Inducible Factor 1-Alpha and Inducible Nitric Oxide Synthase.   *Circulation* 108 (supplement): IV-510, 2003

13. Okubo S, Fisher PW, **Salloum F**, Ockaili R, Marwaha VR, Ambrosio G, Hess ML, Kukreja RC. Angiotensin II Receptor Blockade with Losartan Reduces Apoptosis and Necrosis Ischemia/Reperfusion Injury in the Rabbit Heart. *JACC* 2005 45(3) Supplement A: p252A.

14. Fisher PW, **Salloum F**, Das A, Kukreja RC. Type-5A Phosphodiesterase Inhibition Using Sildenafil (Viagra) Attenuates Cardiomyocyte Apoptosis and Left Ventricular Dysfunction in a Chronic Model of Doxorubicin-induced Cardiotoxicity. J Mol Cell Cardiol 2005. In Press

Technical/Laboratory Skills

1. Animal Surgery including mouse, rat, and rabbit models of ischemia/reperfusion injury

2. Established mouse model of ischemia/reperfusion injury via Langerdorff Mode

3. Western Blot Analysis

4. Hemodynamic analysis of mouse hearts via Isolated Langerdorff Method

5. Isolation of adult mouse/rat ventricular cardiomyocytes for *in vitro studies*