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Long-term cardioprotection with phosphodiesterase-5 inhibition against ischemia-reperfusion injury: Role of nitric oxide.

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LONG-TERM CARDIOPROTECTION WITH PHOSPHODIESTERASE-5 INHIBITION AGAINST ISCHEMIA-REPERFUSION INJURY: ROLE OF NITRIC OXIDE

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

by

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Table 1: Abbreviated terms.

5-HD	5-hydroxydecanoate
cGMP	Cyclic guanosine monophosphate
HR	Heart rate
I/R	Ischemia-reperfusion
LAD	Left anterior descending coronary artery
L-NAME	N_{ω}-Nitro-L-arginine methyl ester hydrochloride
MAP	Mean arterial pressure
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
PC	Preconditioning
PDE-5	Phosphodiesterase-5
PKG	Protein kinase G
RPP	Rate-pressure product
RTPCR	Reverse transcriptase polymerase chain reaction
Sil	Sildenafil
TTC	2,3,5-triphenyltetrazolium chloride
Var	Vardenafil

Abstract

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Major Director: Rakesh C. Kukreja, Ph.D.
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Recent studies have shown that the potent phosphodiesterase-5 (PDE-5) inhibitor, sildenafil citrate, induces a powerful cardioprotective effect against ischemia-reperfusion (I/R) injury in rabbit and mouse hearts. However, the effect of this drug in inducing long-term protection against I/R injury remains unknown. The goal of this study was to identify the duration of the protective window of sildenafil citrate as well as vardenafil, a more potent PDE-5 inhibitor. Rabbits were treated with sildenafil (0.7 mg/kg, *iv*), vardenafil (0.143 mg/kg), or an equivalent volume of saline. After 24 hrs, 48 hrs, 96 hrs, or 7 days of

sildenafil treatment, the hearts were subjected to I/R. In the vardenafil groups, the hearts were subjected to I/R at 24 hrs and 7 days after administration of the drug. To evaluate the role of nitric oxide (NO) in cardioprotection, a non-selective blocker of nitric oxide synthase, L-NAME (15 mg/kg, *iv*) was administered 10 minutes prior to I/R. The results show significant reductions in infarct size in hearts treated with sildenafil and vardenafil as compared to the corresponding saline controls at all time points. The protective effects of sildenafil and vardenafil were abrogated in animals treated with L-NAME. L-NAME had no effect on infarct size in saline treated control rabbits. These data suggest that both sildenafil and vardenafil induce a long-term protective effect against myocardial infarction which is mediated via a NO-dependent pathway. These studies are important in exploiting the clinical potential of PDE-5 inhibitors in terms of protection against ischemia/reperfusion injury in patients with coronary artery disease.

INTRODUCTION

Restoration of blood flow (reperfusion) to a previously ischemic organ or tissue is a necessary step in the recovery process; however, there is a caveat. Reperfusion can further propagate an injury and ultimately result in myocardial cell death. A reduction in the amount of viable myocardium leads to an inevitable decrease in the ability of the heart to perform its primary function: the pumping of blood to both the systemic and pulmonary circuits. Clearly, preserving any myocardial tissue after an ischemic insult is beneficial in the clinical realm. Such implications could have profound effects on the lives of many individuals who suffer from the various cardiovascular problems in our present time.

Ischemic preconditioning is a cardioprotective mechanism against myocardial-cell death and cardiac dysfunction that results from the reperfusion of an ischemic heart (29). Murry *et al* used brief periods of ischemia to evaluate a potential preconditioning effect in the canine heart (26). These authors demonstrated that a brief episode of ischemia slowed the rate of ATP depletion that occurs during subsequent ischemic episodes. It was also shown that periods of intermittent reperfusion may be beneficial to the myocardium in that they washed out catabolites that tend to accumulate during an ischemic event. They found that ischemic preconditioning resulted in a significant reduction of infarct size within the at-risk area of the canine heart by an average of 25% when compared to controls (26).

Studies in both the rabbit and the canine heart have demonstrated that the cardioprotective effect of ischemic preconditioning appears in two phases: an acute or classical phase which is detectable within minutes after brief episode(s) of ischemia and reperfusion and the delayed phase, which appears 24 hours following the initial ischemic insult and has been shown to last up to 96 hrs (21, 24). Classical forms of preconditioning have limited significance in the clinical realm due to the narrow time window within which the myocardium is actually protected. However, delayed preconditioning is relevant clinically in that it follows approximately 24 hours after the insult and tends to last much longer than classical preconditioning. The delayed phase of preconditioning can be produced not only via sub-lethal ischemia, but also by heat shock (10) and various other endogenously released triggers such as adenosine (3), norepinephrine (2), bradykinin (20), oxygen radicals (7) and opioids (35). It has been widely accepted that the delayed cardioprotective window induced by said pharmacological agents is mediated by an increase in NO production (4, 45). Moreover, NO may modulate K_{ATP}-channel sensitivity to intracellular ATP (38). Several studies have shown the importance of mitochondrial K_{ATP} channels in cardioprotection (8). Using diazoxide, a mitochondrial K_{ATP} channel opener, Garlid *et al* were able to produce significant cardioprotective effects in an isolated, perfused heart (14). Furthermore, Liu *et al* found a substantial reduction in the rate of cell death following simulated ischemia in adult ventricular myocytes (23). The protection provided by the mitochondrial K_{ATP} channels was confirmed when the cardioprotective

effects were abolished upon the addition of 5-hydroxydecanoate (5-HD), a selective mitochondrial K_{ATP} channel blocker (23).

It is clear from the brief review above that extensive research and publications in the field of ischemic preconditioning have vastly extended our understanding of the mechanisms underlying the pathogenesis of I/R injury. There can be little doubt that the elucidation of the pathophysiology and cellular mechanisms of the phenomenon of ischemic preconditioning have enabled us to learn a means of protecting the myocardium in the experimental setting. This evolving field, however, has so far failed to provide any direct evidence that this plethora of experimental and clinical research may one day translate into a clinical reality that would ultimately benefit patients with coronary artery disease. Despite this, the knowledge gained as a result of this research has provided us with tools for protecting myocytes and has enabled us to identify several classes of pharmacological agents that may be able to mimic the protection conferred by ischemic preconditioning. These include agents aimed at triggering the preconditioning phenomenon such as adenosine or its more selective analogues, bradykinin/angiotensin-converting enzyme inhibitors and opioids, and those that target the putative distal mediator of preconditioning (mito-K_{ATP} channels), such as nicorandil.

In the quest for novel drugs in preconditioning, Dr. Kukreja and colleagues used phosphodiesterase-5 (PDE-5) enzyme inhibitors for cardioprotection. PDE-5 inhibitors are the class of drugs used for the treatment of erectile dysfunction (ED) in men. Sildenafil citrate (Viagra®) is one such drug approved for ED (5, 18). It has a proven record of safety in humans as predicted by the results of extensive pharmacological and toxicological

testing in animals and *in vitro.* This has been confirmed by pharmacokinetic exposure data as well (1). Sildenafil selectively inhibits PDE-5, the enzyme that catalyzes the breakdown of cyclic-GMP, a potent, endogenous, smooth muscle relaxant. Sildenafil acts by competing with cGMP for the active site on PDE-5 (9). Studies have shown that sildenafil also serves to enhance nitric oxide (NO)-driven cGMP accumulation in the corpus cavernosum of rabbits without affecting the formation of cyclic-AMP. In the absence of a NO drive, sildenafil potentiated the relaxing effects of NO in the isolated corpus cavernosum, but had no functional effect on said tissues isolated from both the human and the rabbit (32). Many of the biological actions of NO occur via the activation of soluble guanylyl cyclase (GC) and the resulting increase in cGMP tissue levels. Moreover, cGMP has been shown to exert a number of actions that would be expected to be beneficial during myocardial ischemia (44, 22, and 37). According to studies by Kodani *et al*, the increased synthesis of cGMP is necessary in order to produce cardioprotection in delayed ischemic PC in the rabbit heart (19).

Vardenafil is the active ingredient in another PDE-5 inhibiting drug for ED called Levitra®. Like sildenafil, vardenafil binds to the active site on the PDE-5 enzyme, thereby inhibiting the binding of cGMP and thus preventing the breakdown of cGMP (9). For cardioprotection studies, it was hypothesized that the mild vasodilatory effect of sildenafil could potentially release agents such as adenosine, bradykinin, or nitric oxide, which may in turn trigger a preconditioning-like effect in the myocardium (28). Studies have shown that, in the rabbit heart, sildenafil induced both acute and delayed cardioprotective effects, both of which were occurring through a pathway dependent on the opening of

mitochondrial K_{ATP} channels (28). Salloum *et al* have further demonstrated that sildenafil causes an increase in endothelial (eNOS) and inducible NO synthase (iNOS) in the mouse heart and that the magnitude of increase was more pronounced for iNOS than for eNOS (32). A direct protective effect of sildenafil was also shown against necrosis and apoptosis in cardiomyocytes through a NO-signaling pathway (11). Furthermore, Das *et al* found that sildenafil enhanced mRNA and protein content of both iNOS and eNOS. In the same study, myocytes treated with sildenafil prior to simulated ischemia and reoxygenation showed a significant increase in the anti-apoptotic protein, Bcl-2. The increased expression of Bcl-2 was inhibited by L-NAME, an inhibitor of nitric-oxide synthases. It was also suggested that sildenafil may trigger a signaling cascade that involves the generation of NO and the accumulation of cGMP in the myocytes through an eNOS- and iNOS-dependent pathway. This would therefore lead to the opening of the mitochondrial K_{ATP} channels and thus allow for the observed cardioprotective effects (11).

Goals of the Study

Although previous studies from our laboratory have shown the protective effect of sildenafil against I/R injury up to 24 hrs after treatment, it is unclear whether sildenafil or vardenafil have a long lasting protective effect extending beyond the 24-hour period. The current study was designed to identify the window of cardioprotection induced by these drugs *in vivo*. Since NO was observed to be an essential component of delayed protection at 24 hrs with sildenafil (32), we were interested in further identifying the role of NO in long-term protection with PDE-5 inhibitors. Accordingly, we addressed the following two questions in the present study:

1. To determine the time course of the cardioprotective effect of sildenafil and vardenafil in the rabbit model of ischemia/reperfusion injury.
2. To show the role of NO in inducing a cardioprotective effect during long-term protection with sildenafil and vardenafil.

Our results show that both drugs reduced infarct size following I/R injury, which was observed up to 7 days and possibly even longer after the treatment. Moreover, the non-selective nitric oxide synthase inhibitor, L-NAME (N_{ω}-Nitro-L-arginine methyl ester hydrochloride), blocked the protective effect, thus confirming the role of a NO-dependent pathway.

MATERIALS AND METHODS

Animals

Male New Zealand White rabbits, obtained from Blue and Gray Rabbitry (Unionville Lane, Virginia), were used in this study. The rabbits were weighed (2.5 kg to 3.5 kg) and the values were recorded directly on the animals' ear for proper identification. The care and use of the animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Virginia Commonwealth University and the National Institutes of Health (NIH) *Guides for Care and Use of Laboratory Animals* [DHHS Publication No. (NIH) 80-23; Office of Science and Health Reports, Bethesda, MD 20205]. Each group was comprised of 6 animals.

Drug Preparation and Administration

Sildenafil citrate (Viagra®) tablets were crushed and ground to a fine powder using a ceramic mortar and pestle. The compound was then weighed and dissolved in 3 cc of sterile 0.9% saline solution (0.7 mg/kg sildenafil citrate). The drug was administered intravenously through left or right ear via the marginal vein. Depending on the experimental group, the rabbits were allowed to remain for 24, 48, 96, or 168 hours (7 days) prior to undergoing the surgery protocol as described below. L-NAME (15 mg/kg) was given to the appropriate groups intravenously 10 minutes before ischemia. A pure compound of vardenafil HCl was weighed, with 1 mg of the compound being dissolved in

7 ml of 0.9% saline. Each animal received 0.143 mg/kg of vardenafil. The drug was given intravenously through the marginal vein in the ear.

Surgical Procedure

The rabbits were given a 1.5 cc injection (intramuscularly) that contained ketamine HCl (35 mg/kg), xylazine (5 mg/kg), and atropine. The atropine was administered in order to sustain an elevated heart rate throughout the entire surgical protocol. Subsequent doses of anesthetic were given at 40 minute intervals in order to maintain appropriate levels of surgical anesthesia.

A ventral midline incision was made in the neck. A tracheotomy was performed, followed by intubation of the animal. The animals were ventilated mechanically with a positive-pressure ventilator and oxygen was administered via the ventilator as well. The left carotid artery was dissected and cannulated with a polyethylene (PE) catheter and filled with saline and heparin for continuous arterial pressure and hemodynamic monitoring. The right jugular vein was also cannulated with a PE catheter containing saline with heparin for continuous infusion of 0.9% saline solution. In some cases, electrocardiographic leads were attached to subcutaneous electrodes in order to monitor either limb lead II or lead III. Baseline values for systolic and diastolic blood pressure were recorded, as well as values for mean arterial pressure and heart rate.

After hemodynamic stabilization was achieved, a left thoracotomy was performed via the fourth intercostal space. The pericardium was then opened in order to gain access to the heart. Hemodynamic data were recorded again after the thoracotomy and just prior to the induction of ischemia. A 5-0 silk suture equipped with an atraumatic needle was

used to circumvent the left anterior descending (LAD) coronary artery at the halfway point between the atrio-ventricular groove and the apex of the heart. The needle was cut from the suture and the two ends were passed through a vinyl tube that had previously been heated and slightly melted so as to not cause damage to the myocardial tissue and to form an effective snare. The smooth end of the snare was pressed securely against the heart and fixed in place with a hemostat in order to occlude the vessel (Figure 1). Ischemia in the myocardium was confirmed either by direct visualization of the tissue *in situ* due to the presence of regional cyanosis, ST elevation and subsequent depression on the electrocardiogram, or T wave inversion on the electrocardiogram. After 30 minutes of ischemia, blood pressure and heart rate values were recorded, and the snare was released. The suture remained in place although it was no longer preventing blood flow within the LAD. The area was gently massaged with a cotton-tipped applicator to promote regional blood flow. Reperfusion of the heart was allowed to occur for 180 minutes. Reperfusion was confirmed by hyperemia in the regions of the myocardium that were previously cyanotic. Pressure and heart rate measurements were taken every 60 minutes during the reperfusion phase of the protocol. To prevent desiccation, the thoracic cavity was covered with a piece of saline-soaked gauze.

Upon completion of the I/R protocol, the heart was removed and mounted on a Langendorff apparatus. The coronary arteries were perfused with a 0.9% saline solution containing 2.5 mM $CaCl_2$ in order to wash out any blood that remained. The suture around the LAD was tied off completely and about 2 ml of 10% Evan's blue dye were injected into the aorta until most of the heart turned blue. The excess Evan's blue dye was washed

away by perfusing the heart with more saline. The heart was then removed from the Langendorff apparatus, placed into a Petri dish, and moved to a freezer for approximately 24 hours to facilitate the next step in the protocol. The frozen heart was then cut into approximately 6 transverse slices of equal thickness, starting from the apex and ending at the base. The slices were then incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution of isotonic pH 7.4 phosphate buffer at 37°C for 20 minutes. The TTC reacts with NADH in the presence of dehydrogenase enzymes, causing the viable cells to stain with a deep red color and the dead myocardial tissue to remain pale in color. This allowed for easy differentiation between viable tissue and infracted gray or white necrotic tissue. Following the staining with TTC, the slices were fixed with a 10% formalin solution. The at-risk area was determined using negative staining with the Evan's blue dye. The portions of tissue that were not at risk from coronary occlusion stained a deep blue color while the at-risk regions did not. Computer morphometry (Bioquant imaging software – BIO98) was used to measure the area of infracted tissue, the risk zone, and the whole left ventricle. Infarct size has been expressed as a percentage of the ischemic risk area.

Statistical Analysis

All measurements of infarct size and risk areas are expressed as group means ± SEM. Changes in hemodynamics and infarct size variables were analyzed by two-way repeated-measures 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 a *t*-test. Statistical differences were considered significant if the P value was <0.05.

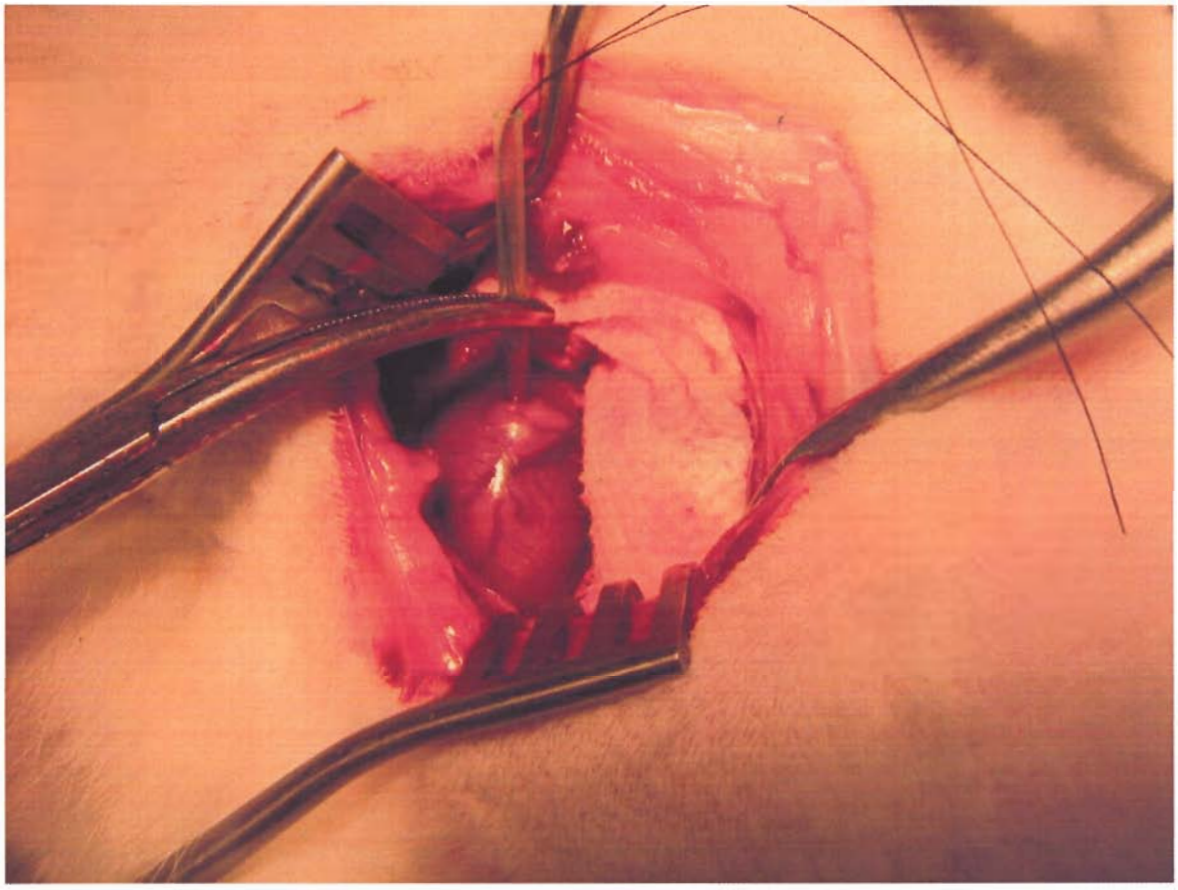

Figure 1. Snare placement and infarction. After the left thoracotomy was performed, the LAD was identified and a 5-0 silk suture passed around it. The two ends of the thread were passed through the tubing and the snare was fixed in place via a hemostat. Confirmation of successful occlusion was achieved by noting regional cyanosis, as seen in this photograph.

RESULTS

Risk Area and Infarct Size

The risk areas for all of the hearts were similar in size, ranging from 46.20% ± 3.48 to 58.05% ± 6.78 (Figures 2, 4 and 6). 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. In figure 3, it is clear that the group with the smallest infarct size (% of risk area) was the one receiving sildenafil from 48-168 hrs prior to I/R. Overall, it can easily be seen that the groups receiving sildenafil, regardless of the time interval, had much smaller infarcts when compared to the control groups (Figure 3).

As seen in figure 5, the addition of L-NAME abolished the protection conferred by sildenafil. The animals receiving sildenafil 24 hrs prior to I/R had an average infarct size of 19.77% ± 1.46, which was increased to 30.77% ± 1.83 in L-NAME treated rabbits. Such increase in the infarct size in sildenafil + L-NAME treated animals was not different when compared with saline treated controls (35.89% ± 1.54). Similarly, the group of animals receiving sildenafil 168 hrs prior to I/R had an infarct size of 16.37% ± 1.94 which increased to 31.61% ± 1.22 following treatment with L-NAME. Again this increase in infarct size was not significantly different as compared to the saline treated control animals, which had an average infarct size of 37.28% ± 1.39 (Figure 5). L-NAME alone

had an infarct size of 34.9 % ± 0.91, which was not significantly different when compared to the saline treated control groups 35.89 % ± 1.54 (24 hrs) or 37.28 % ± 1.39 (168 hrs) subjected to ischemia/reperfusion protocol.

In figure 7, the groups receiving vardenafil showed significant protection at 24 hr and 168 hr (7 days) time intervals. The infarct size was 18.31 ± 1.73 and 22.09 ± 1.12 in the vardenafil-treated rabbits, which was significantly lower as compared to the infarct sizes of 33.4% ± 1.03 and 34.8% ± 1.61 in the saline treated controls at 24 and 169 hrs respectively. Similar to sildenafil, treatment with L-NAME abolished the cardioprotective effect of vardenafil. The infarct size increased to 32.31% ± 2.24 at 24 hrs and 31.24% ± 1.51 at 168 hrs following treatment with L-NAME. The infarct sizes in vardenafil + L-NAME treated groups were comparable to the saline-treated controls: 33.4% ± 1.03 and 34.8% ± 1.61 at 24 and 168 hrs after treatment, respectively (Figure 7).

Hemodynamic Findings

The changes in hemodynamics immediately following treatment with sildenafil and vardenafil have been reported previously from the laboratory (28, 31). Intravenous administration of sildenafil citrate induced a rapid decrease in the hemodynamics as demonstrated by the 24.5%, 47.3%, and 38.8% decline in systolic, diastolic, and mean arterial pressures, respectively, within 2 min. The systemic hemodynamics bounced back to nearly baseline levels within 5 min after treatment with sildenafil. No significant changes in heart rate were observed. Similarly, after treatment with vardenafil, the mean arterial blood pressure decreased from 93.5 ± 2.6 to 82.2 ● 1.5 mm Hg and heart rate

(beats/minute) increased from a baseline value of 151 ± 20 to 196 ± 4.6 (mean ± SEM, $p<0.05$) within 5 minutes of drug administration.

During I/R, the heart rate, MAP and RPP are shown in Table 1 (for sildenafil and controls), Table 2 (for sildenafil + L-NAME) and Table 3 (for vardenafil and vardenafil + L-NAME). There was no significant difference in the baseline levels of these parameters between the different groups. Moreover, the heart rate, MAP and RPP remained reasonably stable throughout the reperfusion period, though there was a gradual decrease in all of the groups during this time. Except at the indicated time points, the mean values were not significantly different between the groups at any time point. Significant decreases in heart rate were observed in the groups treated with L-NAME. Also, the rate-pressure product was found to be significantly lower in the L-NAME treated groups as compared to the other groups during I/R.

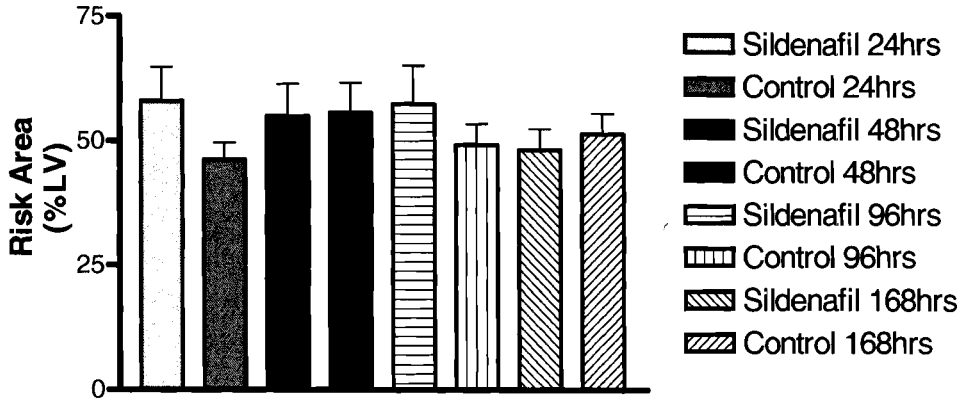

Figure 2. Risk area for long-term sildenafil treatments and controls. The risk area of the left ventricle was determined using Evan's blue dye.

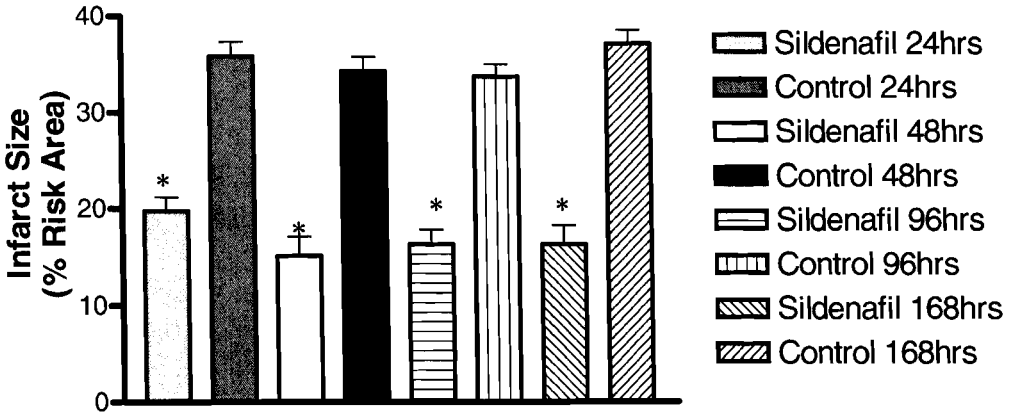

Figure 3. Infarct size of long-term sildenafil treatments and controls. TTC staining was used to assess the amount of viable tissue remaining in the myocardium. Infarct size is expressed as a percentage of the at-risk tissue that suffered from infarction. Control groups received saline only.

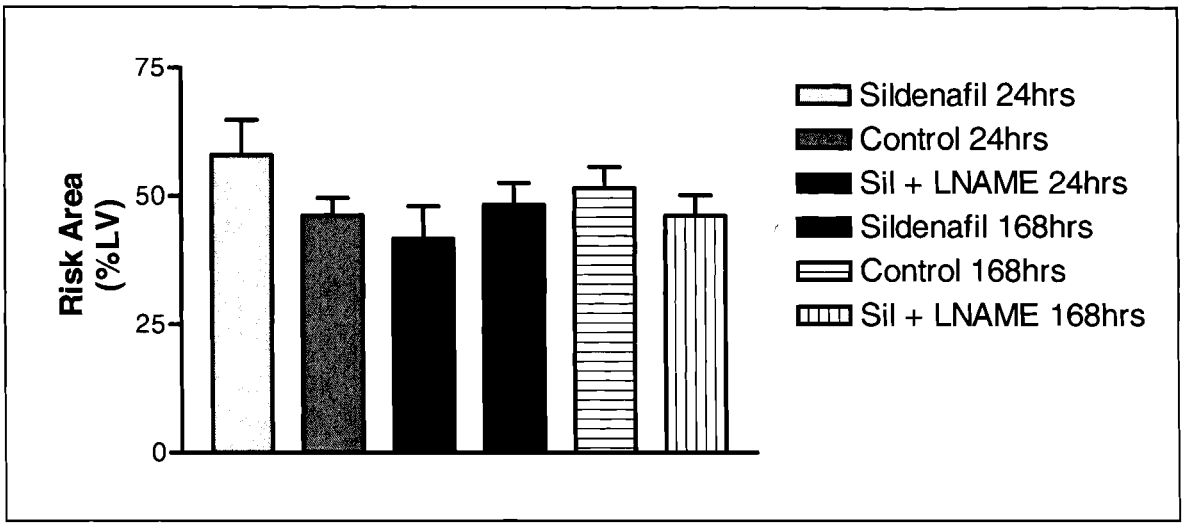

Figure 4. Risk area for sildenafil treatments and L-NAME. The L-NAME, a NO-synthase inhibitor, was given 10 minutes prior to ischemia. Evan's blue dye was used to determine the at-risk area of the left ventricle.

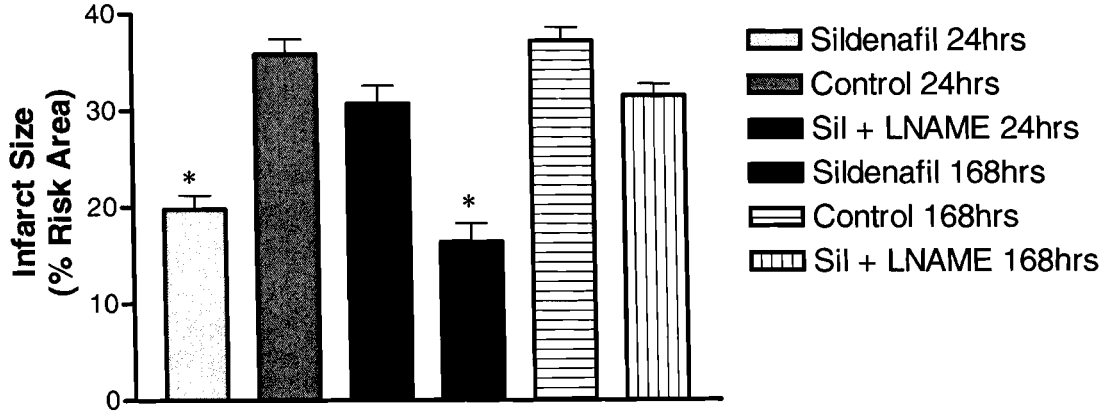

Figure 5. Infarct size of sildenafil treatments and L-NAME. L-NAME was given 10 minutes prior to ischemia-reperfusion. TTC staining was used to assess the amount of viable tissue present within the risk area.

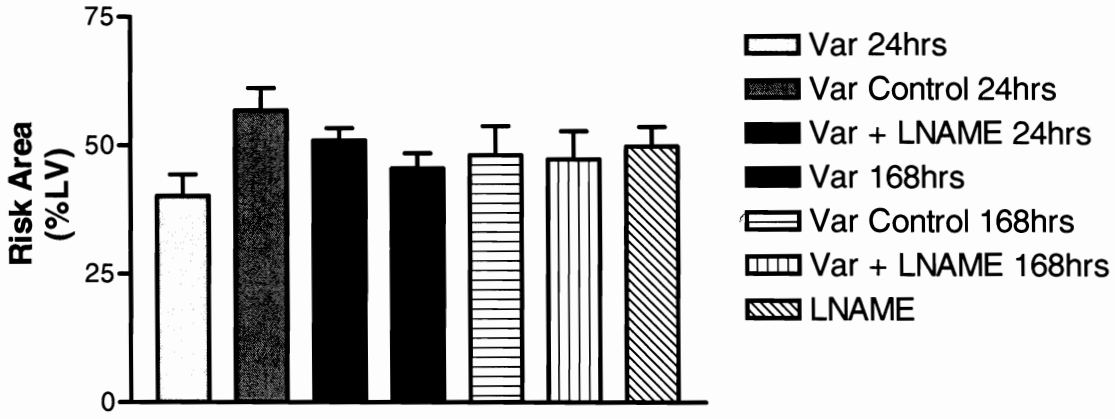

Figure 6. Risk area for delayed and long-term vardenafil treatments and controls. The risk area of the left ventricle was determined using Evan's blue dye. L-NAME was given 10 minutes prior to I-R.

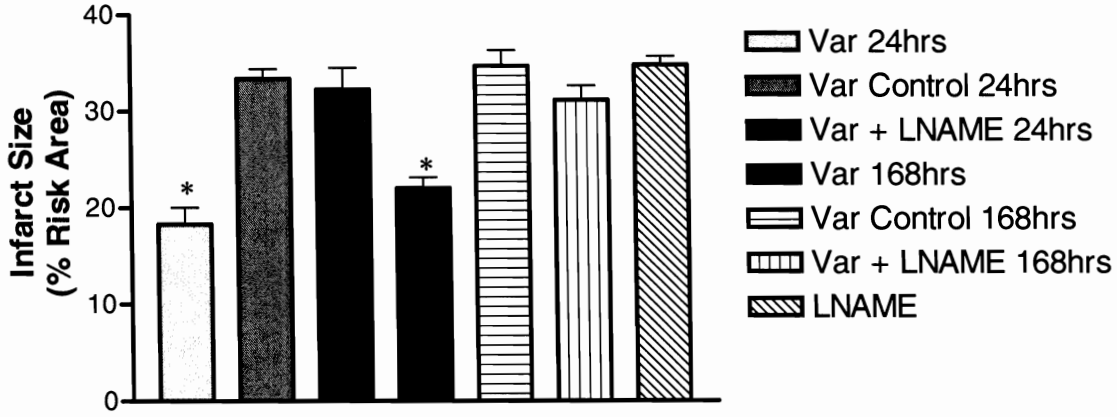

Figure 7. Infarct size of vardenafil treatments and L-NAME. L-NAME was given 10 minutes prior to ischemia-reperfusion. TTC staining was used to assess the amount of viable tissue present within the risk area.

Table 2: Hemodynamic data for sildenafil groups and saline controls

Group	*Baseline*	*Pre-Ischemia*	*30-min Ischemia*	*60-min Reperfusion*	*120-min Reperfusion*	*180-min Reperfusion*
Sil 24 hrs (Group I)						
HR	182 ± 15	190 ± 14	169 ± 6	143 ± 10[f]	126 ± 12[e,f]	135 ± 13[e,f]
MAP	84 ± 2	80 ± 2[a]	66 ± 3[e,f]	64 ± 3[e,f]	66 ± 5[e,f]	61 ± 5[e,f]
RPP	17709 ± 2217	17248 ± 1708	12937 ± 887[e,f]	10575 ± 732[e,f]	9110 ± 976[e,f]	9639 ± 1003[e,f]
Saline Control 24 hrs (GroupII)						
HR	187 ± 9	191 ± 14	177 ± 12	182 ± 13	168 ± 13	164 ± 15
MAP	100 ± 7[a]	96 ± 6[c]	79 ± 3[a,c,e]	80 ± 3[b,c,e]	75 ± 3[e,f]	70 ± 6[e,f]
RPP	10482 ± 1762[a,b,c]	9671 ± 1128[a,b,d]	6545 ± 495[a,c,d,e]	6669 ± 469[a,c,d,e]	5918 ± 526[a,c,d,e,f]	5208 ± 736[a,c,d,e,f]
Sil 48 hrs (Group III)						
HR	177 ± 11	189 ± 8	187 ± 7	158 ± 11	158 ± 14	150 ± 15
MAP	85 ± 5	83 ± 2	72 ± 4	69 ± 5[e]	65 ± 4[e,f]	62 ± 4[e,f]
RPP	15528 ± 1500	16852 ± 2046	15821 ± 1425	13345 ± 1952	12859 ± 2121	11178 ± 1864
Saline Control 48 hrs (Group IV)						
HR	206 ± 12	205 ± 11	185 ± 13	193 ± 11[a,b]	190 ± 12[b]	190 ± 12[b]
MAP	98 ± 8[a]	95 ± 5[a,c]	83 ± 4[a,b,c]	78 ± 4[c]	73 ± 5[e,f]	71 ± 6[e,f]
RPP	10260 ± 1872[a,b,c]	9503 ± 1015[a,b,d]	7161 ± 752[a,c,d]	6380 ± 660[a,c,d]	5678 ± 823[a,c,d,e]	5217 ± 816[a,c,d,e]
Sil 96 hrs (Group V)						
HR	163 ± 18	186 ± 9	195 ± 7	168 ± 10	159 ± 14	154 ± 14
MAP	85 ± 3	77 ± 3	59 ± 4[e,f]	55 ± 3[e,f]	59 ± 6[e,f]	58 ± 6[e,f]
RPP	16566 ± 2253	16759 ± 1345	14122 ± 1109	11464 ± 1042	11628 ± 1603	11040 ± 1535
Saline Control 96 hrs (Group VI)						
HR	181 ± 7	187 ± 9	188 ± 8	181 ± 9	174 ± 11	170 ± 13
MAP	97 ± 4	94 ± 4[a,c]	78 ± 2[e,f]	75 ± 2[e,e,f]	71 ± 2[e,f]	64 ± 4[e,f]
RPP	9680 ± 722[a,b,c]	9222 ± 863[a,b,d]	6342 ± 364[a,c,d,e,f]	5868 ± 378[a,c,d,e,f]	5277 ± 212[a,c,d,e,f]	4362 ± 409[a,c,d,e,f]
Sil 7 days (Group VII)						
HR	198 ± 15	201 ± 14	197 ± 8	148 ± 10[e,f,g]	145 ± 8[e,f,g]	141 ± 6[e,f,g]
MAP	75 ± 5	74 ± 5	65 ± 3	69 ± 4	69 ± 2	66 ± 3
RPP	17180 ± 1043	17265 ± 1038	15905 ± 1132	12044 ± 858[e,f,g]	11513 ± 647[e,f,g]	10773 ± 807[e,f,g]
Saline Control 7 days (Group VIII)						
HR	199 ± 13	201 ± 7	196 ± 7	193 ± 6[a,b]	196 ± 6[a,b]	196 ± 7[a,b]
MAP	94 ± 5	93 ± 4	82 ± 4[a,b,c]	73 ± 3[e,f]	69 ± 4[e,f]	64 ± 4[e,f]
RPP	9458 ± 922[a,b,c]	9054 ± 687[a,b,d]	6959 ± 709[a,c,d,e,f]	5579 ± 516[a,c,d,e,f]	5037 ± 619[a,c,d,e,f]	4365 ± 542[a,c,d,e,f]

Values are means ± SEM. HR – Heart rate (beats/min); MAP – Mean arterial blood pressure (mmHg); RPP – Rate pressure product (mmHg/min). [a]*P<0.05 vs Sil 7 days,* [b]*P<0.05 vs Sil 24hrs,* [c]*P<0.05 vs Sil 96hrs,* [d]*P<0.05 vs Sil 48hrs,* [e]*P<0.05 vs baseline,* [f]*P<0.05 vs pre-ischemia,* [g]*P<0.05 vs 30 min ischemia.*

Table 3: Hemodynamic data for sildenafil, sildenafil + L-NAME and controls

Group	*Baseline*	*Pre-Ischemia*	*30-min Ischemia*	*60-min Reperfusion*	*120-min Reperfusion*	*180-min Reperfusion*
Sil 24 hrs (Group I)						
HR	182 ± 15	190 ± 14	169 ± 6	143 ± 10[c]	126 ± 12[d,e]	135 ± 13[d,e]
MAP	84 ± 2	80 ± 2	66 ± 3[d,e]	64 ± 3[d,e]	66 ± 5[d,e]	61 ± 5[d,e]
RPP	17709 ± 2217	17248 ± 1708	12937 ± 887[d,e]	10575 ± 732[d,e]	9110 ± 976[d,e]	9639 ± 1003[d,e]
Saline Control 24 hrs (Group II)						
HR	187 ± 9	191 ± 14	177 ± 12	182 ± 13	168 ± 13	164 ± 15
MAP	100 ± 7	96 ± 6	79 ± 3[a,d]	80 ± 3[a,d]	75 ± 3[d,e]	70 ± 6[d,e]
RPP	10482 ± 1762[a]	9671 ± 1128[a]	6545 ± 495[d]	6669 ± 469[d]	5918 ± 526[d,e]	5208 ± 736[d,e]
Sil 24 hrs + L-NAME (Group III)						
HR	169 ± 20[c]	144 ± 16	135 ± 11[b,c]	130 ± 8[b,c]	128 ± 10[c]	119 ± 9[c]
MAP	84 ± 8	107 ± 14	84 ± 5[a]	87 ± 3[a,c]	79 ± 3	76 ± 3
RPP	15938 ± 1138	15488 ± 2421	13124 ± 1376[b,c]	12474 ± 907[b,c]	11271 ± 700[b,c]	10015 ± 524[b,c,d]
Sil 7 days (Group IV)						
HR	198 ± 15	201 ± 14	197 ± 8	148 ± 10[c,d,e,f]	145 ± 8[c,d,e,f]	141 ± 6[c,d,e,f]
MAP	75 ± 5[b]	74 ± 5	65 ± 3[b,c]	69 ± 4	69 ± 2	66 ± 3
RPP	17180 ± 1043[b,c]	17265 ± 1038[b,c]	15905 ± 1132[b,c]	12044 ± 858[b,c,d,e,f]	11513 ± 647[b,c,d,e,f]	10773 ± 807[b,c,d,e,f]
Saline Control 7 days (Group V)						
HR	199 ± 13	201 ± 7	196 ± 7	193 ± 6[a]	196 ± 6[a]	196 ± 7[a,b]
MAP	94 ± 5	93 ± 4	82 ± 4[a]	73 ± 3[d,e]	69 ± 4[d,e]	64 ± 4[d,e,f]
RPP	9458 ± 922[a]	9054 ± 687[a]	6959 ± 709[d,e]	5579 ± 516[d,e]	5037 ± 619[d,e]	4365 ± 542[d,e]
Sil 7 days + L-NAME (Group VI)						
HR	182 ± 13	158 ± 11	145 ± 11[c,d]	129 ± 8[b,c,d]	129 ± 8[c,d]	118 ± 8[c,d]
MAP	84 ± 4	91 ± 3	82 ± 4[a]	86 ± 3[a,d]	77 ± 6	74 ± 7
RPP	17508 ± 1453[b,c]	17188 ± 1264[b,c]	14022 ± 1126[b,c,d,e]	12390 ± 690[b,c,d,e]	11859 ± 506[b,c,d,e]	9802 ± 293[b,c,d,e,f]
L-NAME Control (Group VII)						
HR	182 ± 8	191 ± 9	177 ± 13	160 ± 19	155 ± 20[c]	138 ± 15[c]
MAP	85 ± 6	101 ± 23	75 ± 3	70 ± 1	69 ± 1	68 ± 1
RPP	17837 ± 920[b,c]	17426 ± 805[b,c]	14346 ± 1035[b,c]	12609 ± 1184[b,c,d,e]	12286 ± 1397[b,c,d,e]	10761 ± 999[b,c,d,e]

Values are means ± SEM. HR – Heart rate (beats/min); MAP – Mean arterial blood pressure (mmHg); RPP – Rate pressure product (mmHg/min).
[a]P<0.05 vs Sil 24hrs, [b]P<0.05 vs saline 24hrs, [c]P<0.05 vs saline 7 days, [d]P<0.05 vs baseline, [e]P<0.05 vs pre-ischemia, [f]P<0.05 vs 30min ischemia.

Table 4: Hemodynamic data for vardenafil, vardenafil + L-NAME and controls

Group	*Baseline*	*Pre-Ischemia*	*30-min Ischemia*	*60-min Reperfusion*	*120-min Reperfusion*	*180-min Reperfusion*
Vardenafil 24 hrs (Group I)						
HR	182 ± 15	190 ± 14	169 ± 6	143 ±10[f]	126 ± 12[e,f]	135 ± 13[e,f]
MAP	84 ± 3	80 ± 2	66 ± 3[e,f]	64 ± 3[e,f]	67 ± 5[e,f]	61 ± 5[e,f]
RPP	17709 ± 2217	17248 ± 1708	12937 ± 887	10575 ± 732[e,f]	9110 ± 976[e,f]	9639 ± 1003[e,f]
Saline Control 24 hrs (Group II)						
HR	187 ± 9	191± 14	177 ± 12	182 ± 13	168 ± 13	164 ± 15
MAP	100 ± 7	96 ± 6	79 ± 3[e]	80 ± 3[e]	75 ± 3[e,f]	70 ± 6[e,f]
RPP	10482 ± 1762[a]	9671 ± 1128	6545 ± 495[a,e]	6669 ± 469[a,e]	5918 ± 526[a,e,f]	5208 ± 736[a,e,f]
Vardenafil 24 hrs + L-NAME (Group III)						
HR	180 ± 17	193 ± 8	149 ± 18	145 ± 18	145 ± 10[d]	134 ± 11[d]
MAP	83 ± 8	107 ± 10	86 ± 8[a,c]	66 ± 10[f]	52 ± 8[b,f]	55 ± 10[f]
RPP	18811 ± 3218[b,d]	23941 ± 3137[a,b,c,d]	14105 ± 1184[b,d,f]	10263 ± 663[e,f]	8674 ± 852[e,f]	8311 ± 1054[e,f]
Vardenafil 7 days (Group IV)						
HR	198 ± 15	201 ± 14	197 ± 8	148 ± 10[e,f]	145 ± 8[e,f]	141 ± 6[e,f]
MAP	75 ± 5	74 ± 5	65 ± 3	69 ± 4	69 ± 2	66 ± 3
RPP	17180 ± 1044	17265 ± 1038[b]	15905 ± 1132[b]	12044 ± 858[b,e,f]	11513 ± 647[b,e,f]	10773 ± 807[b,e,f]
Saline Control 7 days (Group V)						
HR	199 ± 13	201 ± 7	196 ± 7	193 ± 6[e,f]	196 ± 6[a,c,e,f]	196 ± 7[a,c,e,f]
MAP	94 ± 5	93 ± 4	82 ± 4	73 ± 3	69 ± 4	64 ± 4
RPP	9458 ± 922[a]	9054 ± 687[c]	6959 ± 709[a,c]	5579 ± 516[a,c,e,f]	5037 ± 619[a,c,e,f]	4365 ± 542[a,c,e,f]
Vardenafil 7 days + L-NAME (Group VI)						
HR	189 ± 8	171 ± 11	131 ± 13[c,e,f]	121 ± 9[b,d,e,f]	117 ± 10[d,e,f]	111 ± 12[b,d,e,f]
MAP	87 ± 13	104 ± 7	82 ± 6	82 ± 1	74 ± 5	72 ± 7
RPP	19593 ± 1488[b,c]	19712 ± 1178[b,c]	11805 ± 993[c,e,f]	11200 ± 908[b,d,e,f]	9792 ± 656[b,d,e,f]	8748 ± 319[b,d,e,f]
L-NAME Control (Group VII)						
HR	182 ± 8	191 ± 9	177 ± 13	160 ± 19	155 ± 20	138 ± 15[d]
MAP	85 ± 6	101 ± 23	75 ± 3	70 ± 1	69 ± 1	68 ± 1
RPP	17837 ± 920[b,d]	17426 ± 805[b,d]	14346 ± 1035[b,d]	12609 ± 1184[b,d,e,f]	12286 ± 1397[b,d,e,f]	10761 ± 999[b,d,e,f]

Values are means ± SEM. HR – Heart rate (beats/min); MAP – Mean arterial blood pressure (mmHg); RPP – Rate pressure product (mmHg/min).
[a]P<0.05 vs Var24hrs, [b]P<0.05 vs saline 24hrs, [c]P<0.05 vs Var 7 days, [d]P<0.05 vs saline 7days, [e]P<0.05 vs baseline, [f]P<0.05 vs pre-ischemia.

DISCUSSION

Ischemic heart disease, as the underlying cause of most cases of acute myocardial infarction (AMI), congestive heart failure, arrhythmias, and sudden cardiac death, is the leading cause of morbidity and mortality in all industrialized nations. In the United States, ischemic heart disease causes nearly 20% of all deaths (approximately 600,000 deaths each year), with greater than half of these deaths occurring before the patient arrives at the hospital, primarily due to arrhythmias and cardiac arrest. An estimated 1.1 million Americans will have a new or recurrent AMI this year, and many survivors will experience lasting morbidity, with progression to heart failure and death. As the population grows older and co-morbidities such as obesity and diabetes become more prevalent, the enormous public health burden caused by ischemic heart disease is likely to increase even more. Two critical factors are required in order to improve the outcome of a patient suffering from an acute, ischemic event. First, the patient must survive any arrhythmias. The majority of deaths due to AMI occur prior to hospitalization, due primarily to arrhythmias and cardiac arrest. Although greater access to automatic defibrillators and optimization of CPR protocols offer tremendous potential to save lives, it is remarkable that survival is still dependent upon early CPR and rapid defibrillation. Indeed, survival rates from cardiac arrest have shown only marginal improvements over the last 30 years,

which further underscores the need for novel therapies and resuscitation strategies. Second, infarct size needs to be limited. For patients with AMI who do not succumb to out-of-hospital arrhythmias and are successfully transported to the hospital, the prognosis is dependent on the amount of myocardium that is lost as a result of ischemia/reperfusion injury. There is no question that timely reperfusion (by thrombolysis or percutaneous transluminal coronary angioplasty [PTCA]) can salvage ischemic myocardium – and has, indeed, become the standard in-hospital treatment for AMI. Although greater benefits can conceptually be achieved by continued efforts to implement even earlier restoration of coronary flow, delays in seeking medical attention, together with inherent logistic and temporal limitations in initiating thrombolysis or PTCA, make it unlikely that additional and substantive improvements in morbidity and mortality can be achieved by reperfusion therapy alone.

Limitation of myocardial ischemia/reperfusion injury is also of paramount importance in the setting of global myocardial ischemia associated with coronary artery bypass graft (CABG) surgery. Despite the considerable progress that has been made to date, high-risk subsets of patients (i.e., repeat CABG, unstable angina, LV dysfunction, diabetes, old age, etc.) continue to exhibit post-operative complications, including prolonged contractile dysfunction (stunning), low-output syndrome, peri-operative myocardial infarction, and cardiac failure requiring prolonged intensive care. Thus, both in patients experiencing an AMI and in those undergoing CABG surgery, there is a compelling, but still unfulfilled, need to protect the ischemic myocardium.

In recent studies from Dr. Kukreja's laboratory, it was shown that PDE-5 inhibitors sildenafil (Viagra®) and vardenafil (Levitra®) induce preconditioning-like protective effects against ischemia-reperfusion injury in adult rabbit hearts. Sildenafil administered 30 minutes (acutely) or 24 hours (delayed) prior to ischemia-reperfusion has previously been shown to be an effective means for cardioprotection in the rabbit heart (28). However, the efficacy of these drugs in causing long-term protection following ischemia/reperfusion injury has not been investigated. In this study, we wanted to test if sildenafil could outlast the second window of protection seen in classical ischemic preconditioning. Therefore, along with 24-hour delayed protection studies with sildenafil, we administered sildenafil 48 hrs, 96 hrs, and 7 days prior to undergoing the ischemia-reperfusion protocol. We found significant protection at all time points. At 48 hrs, we observed reduction in infarct size from 34.38% ± 1.46 in saline controls to 15.15% ● 2.01 with sildenafil treatment. On day 7 after administration of sildenafil, the infarct size was reduced to 16.37% ± 1.94 as compared 37.28% ± 1.39 in saline control ($p < 0.05$). Furthermore, we observed a similar reduction in infarct size with vardenafil when administered 24 hrs or 7 days prior to ischemia/reperfusion. This confirms our hypothesis that PDE-5 inhibitors have a "class effect" in terms of long-term cardioprotection.

Transient decreases in systemic pressures have been reported as acute responses to both drugs. For example, Ockaili *et al* (28) reported that intravenous administration of sildenafil caused a rapid decrease in hemodynamics as indicated by the 24.5%, 47.3%, and 38.8% decline in systolic, diastolic, and mean arterial pressures, respectively, within 2 min of receiving the drug. The systemic hemodynamics returned to nearly baseline levels

5 min after treatment with sildenafil. The effect of orally administered sildenafil citrate on systemic hemodynamics was milder and slower compared with the intravenous dose of the drug. The orally administered sildenafil caused a 9.2%, 12.5%, and 10.3% decrease in systolic, diastolic, and mean arterial pressure, respectively, after 30 min of treatment with the drug. This hypotensive response remained significantly depressed even at 60 min after oral administration of the drug. No changes in heart rate were observed. However, we did not see any changes in baseline hemodynamics 24-168 hours after drug administration. Although L-NAME abolished cardioprotection at all time intervals, suggesting that there must be some up-regulation of iNOS and/or eNOS protein levels following treatment with these drugs, this increase may not be enough to elicit a systemic hypotensive response. Chen *et al* suggested that hypovolemic hypotension has a preconditioning effect in the rabbit heart. They found that hypovolemic hypotension preconditioning significantly improves cardiopulmonary function and increases NO formation and that the protective benefit associated with hypovolemic hypotension preconditioning of the heart may be regulated through NO mediated mechanism (6). Therefore, hypotensive preconditioning could be one of the mechanisms producing a PC-like effect in our long-term studies.

The exact mechanism of long-term protection by PDE-5 inhibition is not fully understood. However our data suggest that NO generated after treatment with sildenafil or vardenafil plays an essential role in long-term protection. Treatment with a non-selective blocker of nitric oxide synthases (L-NAME) abolished the protection conferred by sildenafil as well as vardenafil. The infarct size was increased to the size similar to saline controls (Figures 5 and 7). NO has been shown to play a prominent role both in initiating

and in mediating the cardioprotective response of preconditioning. Several studies from our laboratory have demonstrated that delayed pharmacological PC is mediated by the upregulation of iNOS in the myocardium (40, 46, and 47). Bolli and colleagues have shown that NO is the key trigger as well as a key mediator of PC in rabbit and mouse hearts (4, 16). In the Langendorff isolated perfused mouse heart model subjected to 20 minutes of global ischemia and 30 minutes of reperfusion, pre-treatment of the animals with sildenafil reduced myocardial infarct size 24 hours later compared with saline controls (32).

Sildenafil-induced protection was abolished by a selective iNOS inhibitor, 1400W. In these studies, sildenafil did not alter pre-ischemic or post-ischemic coronary flow, indicating that its cardioprotective effect may be independent of its vascular response 24 hours later. RTPCR 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. The magnitude of increase was much higher for iNOS mRNA as compared with eNOS mRNA. In addition, cardiac expression of iNOS and eNOS protein was significantly elevated 24 hours after sildenafil treatment. These studies suggest that sildenafil induces delayed preconditioning 24 hrs later and that this response is primarily mediated by NO derived from iNOS. Similarly in isolated cardiomyocytes (11), sildenafil caused a significant increase in mRNA and protein expression of iNOS and eNOS (to a lesser extent). Also, sildenafil-induced protection against necrosis and apoptosis was abolished in myocytes deprived of iNOS, but not in eNOS gene knockout mice. Interestingly, sildenafil-treated myocytes demonstrated an

early increase in the Bcl-2/Bax ratio following simulated ischemia-reperfusion, which may have been responsible for the anti-apoptotic effect of sildenafil. The increase of the Bcl-2/Bax ratio, as well as the anti-apoptotic effects of sildenafil, were inhibited by treatment with the NOS inhibitor, L-NAME, thus suggesting the role of NO signaling in the protective effect of the drug against apoptosis. In the present study, the role of NO derived from nitric oxide synthase is clearly indicated by the ability of L-NAME to block the protection; however, we have not identified the specific isoform of the enzyme. As mentioned above, although iNOS is involved in cardioprotection 24 hrs later with sildenafil, we do not know whether the same isoform of the enzyme is implicated in long term protection observed at 48-168 hrs following treatment with sildenafil or vardenafil. Wang *et al* suggested that cardioprotection at 72 hrs after the initial bouts of ischemic preconditioning was mediated by nNOS and cyclooxygenase-2 (42). Surprisingly, the iNOS expression was not increased at 72 hours; however, upregulation of nNOS was evident at both the mRNA as well as protein levels. These changes were accompanied by an increase in myocardial nitrite/nitrate. Furthermore, the nNOS-selective inhibitors N-propyl-l-arginine or S-ethyl N-[4-(trifluoromethyl)phenyl] isothiourea completely blocked the protection of delayed preconditioning at 72 hours, whereas the iNOS-selective inhibitor S-methylisothiourea had no effect (33). It is possible that a similar shift in the source of NO occurs following long-term protection with PDE-5 inhibitors. Future studies will address this important issue.

There are several possibilities by which NO may induce a cardioprotective effect. Enhanced synthesis of cGMP has been shown to be a requirement for the protection in

delayed-ischemic PC in the rabbit hearts (19). In these studies, the role of cGMP did not exist during the early (acute) phase of ischemic PC. It was proposed that NO participates in PC via two distinct mechanisms; it triggered delayed ischemic PC on acutely via a cGMP-independent mechanism and yet it mediated delayed PC 24 hrs later via a cGMP-dependent mechanism. Furthermore, cGMP may activate PKG (protein kinase G), which can subsequently open mitochondrial K_{ATP} channels (17). Conceptually, sildenafil plays a pivotal role in cardioprotection because it inhibits the enzymatic hydrolysis of cGMP and maintains the tissue accumulation of cGMP, thus leading to the downstream protective mechanism that involves the PKG-activated mitochondrial-K_{ATP} channel. In addition, it was proven that myocardial cGMP content was in fact enhanced following the pretreatment rats with the cardioprotective dose of sildenafil (11). Further studies that would prove helpful in this area would be to examine tissue levels of cGMP and NO at the appropriate time intervals. Quantifying those concentrations in the tissues would allow us to determine the thresholds these biochemical mediators must reach in order to achieve a cardioprotective effect with PDE-5 inhibitors in the heart.

The opening of mitochondrial K_{ATP} channels may be one of the important mechanisms of long-term cardioprotection achieved with PDE-5 inhibitors. 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. In the rabbit studies, Ockaili *et al* found that both acute and delayed cardioprotective effects were blocked by 5-HD, suggesting that opening of mitochondrial K_{ATP} channels does in fact play an important role in the infarct size reduction by sildenafil (28). 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 allows for additional protons to be pumped out, forming an H^+ electrochemical gradient for both ATP synthesis and Ca^{2+} transport (39, 15). The delayed PC could be through the signaling cascade leading to the synthesis of iNOS, generation of NO and opening of the mitoK_{ATP} channels as described previously (27, 33, and 43).

In summary, we have shown that a novel class of PDE-5 inhibitors, including sildenafil and vardenafil, can reduce myocardial infarct size following ischemia/reperfusion up to 7 days following treatment. Our results also suggest that such a long lasting protective effect of these drugs is mediated by NO generated from nitric oxide synthase. These studies could be important in terms of harvesting the clinical potential of phosphodiesterase-5 inhibitors for protection of the heart against ischemia/reperfusion injury in patients with coronary artery disease.

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Literature Cited

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