PHOSPHODIESTERASE-5 INHIBITION: A NOVEL STRATEGY TO IMPROVE STEM CELL THERAPY IN THE HEART

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PHOSPHODIESTERASE-5 INHIBITION: A NOVEL STRATEGY TO IMPROVE STEM CELL THERAPY IN THE HEART

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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Abstract

PHOSPHODIESTERASE-5 INHIBITION: A NOVEL STRATEGY TO IMPROVE STEM CELL THERAPY IN THE HEART

By Nicholas N. Hoke, Ph.D.

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

Virginia Commonwealth University, 2011

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Several studies have shown cellular replacement therapy as a treatment strategy of myocardial infarction but results have been limited. Therefore, enhancing the therapeutic potential of stem cells injected into ischemic microenvironments by novel preconditioning (PC) techniques is critical for improving cellular therapy. Recent studies have shown that inhibition of phosphodiesterase-5 (PDE-5) is a powerful strategy to precondition the heart and cardiomyocytes against ischemia/reperfusion injury. We therefore tested the hypothesis that inhibition of PDE-5 with sildenafil (Viagra®) or selective knockdown with a silencing vector in
adipose derived stem cells (ASCs) would improve their survival after ischemia/reoxygenation *in vitro* and enhance cardiac function following myocardial implantation *in vivo*.

ASCs were treated with sildenafil or infected with PDE-5 silencing vector shRNA (shRNA^{PDE-5}). The cells were subjected to simulated ischemia (SI) and reoxygenation (RO). Both sildenafil and shRNA^{PDE-5} significantly reduced cell injury, as shown by improved viability, decreased lactate dehydrogenase, and apoptosis. The preconditioned ASCs also demonstrated an increase in the release of growth factors including VEGF, b-FGF, and IGF. The protective effect against SI/RO injury was abolished by inhibition of protein kinase G (PKG) using both a pharmacological inhibitor and selective knockdown with shRNA^{PKG1α} suggesting a PKG-mediated mechanism. To show the effect of preconditioned ASCs *in vivo*, adult male CD-1 mice underwent myocardial infarction (MI) by occlusion of the left descending coronary artery, followed by direct injection of PBS (control), non-preconditioned ASCs, or preconditioned ASCs (4x10^5) ASCs into the left ventricle (LV). Preconditioned ASC-treated hearts showed consistently superior cardiac function by all measures as compared with PBS and non-preconditioned ASCs after 4 weeks of treatment. Post-mortem histological analysis demonstrated that preconditioned ASC-treated mice had significantly reduced fibrosis, increased vascular density and reduced resident myocyte apoptosis as compared to mice receiving non-preconditioned ASCs or PBS. VEGF, b-FGF, and Ang-1 were also significantly elevated 4 weeks after cell therapy with preconditioned ASCs. Our data suggests that genetic or pharmacological inhibition of PDE-5 is a powerful new approach to improve stem cell therapy following myocardial infarction.
Cardiovascular disease remains a leading cause of morbidity and mortality in the United States, affecting approximately 5–6 million Americans, particularly those of age 65 and older. A continually aging population is expected to result in an increased number of people afflicted with heart-related conditions, requiring costly long-term medical management with an unpredictable effect on quality of life. The most common cardiovascular disease in the United States is coronary heart disease, which often appears as an acute myocardial infarction (MI) caused by the sudden occlusion of the coronary artery. In 2010, an estimated 785,000 Americans had a new coronary attack, and about 470,000 had a recurrent attack. About every 25 seconds, an American will have a coronary event, and about one every minute will die from one (1). Despite advances in treatment of MI that result in reduced mortality, congestive heart failure secondary to infarction continues to be a major complication. MI is projected to remain one of leading causes of death for years to come; therefore, there is a continuous demand for safe and efficient preventive or therapeutic strategies (2).

Etiologies of heart failure development are numerous and involve complex molecular mechanisms, not entirely understood. However, recent advances have expanded our knowledge and understanding of the cellular and molecular mechanisms involved in the development of
heart failure. The occlusion of an artery creates an ischemic microenvironment by depriving areas of the myocardium of blood, oxygen and nutrients, which if maintained for any significant amount of time will trigger a cascade changing the cellular metabolism and function within the tissue primarily from decreased amounts of energy produced in the form of ATP. The lack of sufficient amounts of ATP can eventually lead to severe cellular and tissue damage such as myocyte hypertrophy, myocyte death, and disruption of matrix metalloproteinase balance (3). The degree of injury is dependent on the period for which the myocardium is subjected to ischemia, with a longer duration leading to a decreased chance of recovery (4). Prolonged bouts of ischemia leads to a variety of pathophysiological states such as a decrease in force generation, contracture, arrhythmias, calcium overload, a decrease in pH of the tissue and eventual cell death (5). The loss of contracting myocardium and a resulting increase in the workload on the viable myocardium causes cardiac overload due to increased energy usage and supply-demand imbalance (ATP depletion), which lead to cellular necrosis and apoptosis. This subsequently promotes acute and chronic transformation of both the necrotic infarct zone and the nonnecrotic, peri-infarct tissue, leading to global alterations that have collectively been termed “ventricular remodeling” (6, 7). Progressive cardiac hypertrophy that occurs in response to MI is known to increase the risk of heart failure, although it is believed to be compensatory at the initial stages of remodeling (8). Because of the loss of cardiomyocytes during an MI, the heart, as a result, is unable to maintain a cardiac output appropriate for the requirements of the body.

The main factor leading to the progression of heart failure is the irreversible loss of cardiomyocytes due to necrosis and apoptosis. To overcome myocyte loss and the heart’s limited self-regeneration capacity, mesenchymal stem cell-based therapies are becoming increasingly
recognized for their potential to repair the damaged myocardium post injury. Mesenchymal stem cells are characterized for their self-renewing capacity and ability to undergo multi-lineage differentiation. However, the use of mesenchymal stem cells for the purpose of regenerative medicine should adhere to the following set of criteria: (i) available in abundant quantities; (ii) collected and harvested by a minimally invasive procedure; (iii) differentiated along multiple cell lineage pathways in a reproducible manner; (iv) can be safely and effectively transplanted to either an autologous or allogeneic host (9).

Typically, mesenchymal stem cells are isolated from either bone marrow or adipose tissue. Adipose tissue is an attractive source of mesenchymal stem cells for researchers and clinicians due to the simple surgical procedure, the abundance of subcutaneous adipose tissue, and the easy enzyme-based isolation procedures (10, 11). Adipose tissue-derived stem cells (ASCs) have the ability to differentiate into multiple lineages of tissues, such as skeletal muscle, bone, and fat, using specific culturing conditions containing hormones or growth factors (12-14). Interestingly, ASCs have been shown to differentiate into spontaneous contractile myocytes (15). Transplanted stem cells function through paracrine mechanisms to promote endogenous repair of cardiac tissue (16-18). Moreover, differentiation of ASCs into endothelial cells has been shown to have a strong regenerative angiogenic potential because of their ability to secrete angiogenic and pro-survival paracrine factors (19, 20). Due to their multi-lineage differentiation potential, ASCs are becoming a widely studied alternative to bone marrow-derived stem cells (BMSCs) for therapeutic treatment of cardiac diseases.
Characterization of Adipose Tissue-Derived Stem Cells

Numerous experiments have documented the benefits of BMSCs for treating myocardial infarction (21, 22). Yet, only in the past few years have groups started to study the benefits of cells obtained from adipose tissue for treating heart disease. Interestingly, there are several advantages of using adipose tissue versus bone marrow-derived cells. It is known that ASCs, along with BMSCs, can be maintained in vitro for extended periods of time with a stable population doubling time and low senescence levels, thus implying that there is no deterioration in their proliferation rate (23). Multipotent stem cells from adipose tissue can be harvested from patients by a simple surgical procedure that is less expensive and minimally invasive when compared to obtaining cells from bone marrow. In fact, a greater frequency and yield of multipotent stem cells from adipose tissue was reported when compared to bone marrow isolations, approximately $5 \times 10^5$ cells versus $1 \times 10^5$, respectively (24). Neither the type of surgical procedure nor the anatomical site of the adipose tissue affects the total number of viable cells that can be obtained in the stroma vascular fraction (25). Furthermore, it has been shown that ASCs possess a higher stem cell proliferation rate than BMSCs (24). Moreover, ASCs can be held in culture for up to 1 month without passage while maintaining their proliferation and differentiation potential, thus minimizing time and expenses of tissue culture maintenance. The high yield of isolation, proliferation rate, and maintenance of differentiation potential suggests that it is possible to obtain an autologous line of ASCs from patients undergoing elective liposuction. Cells can also be cryogenically frozen for storage or transplanted either immediately or after expansion in culture if needed for treatment of cardiac injury.
The frequency and yield of ASCs and BMSCs cellular preparations are different, but they share similar biology. It is imperative to determine the gene and protein expression profile of ASCs to improve culturing conditions, lineage-specific differentiation, and enrichment of the stem cell population prior to transplantation. Using fluorescence-activated cell sorting analysis, the surface immunophenotype of ASCs was determined to resemble the phenotypes of BMSCs, stromal cells, or skeletal muscle-derived cells (25-27). Previously reported immunophenotypes of human ASCs and human BMSCs show them to be approximately 90% identical (28). Both cell populations display similar mitogen-activated protein kinase (MAPK) phosphorylation in response to tumor necrosis factor, adiponectin and leptin secretion, and lipolytic response to adrenergic agents (13). Unfortunately, studies comparing the direct gene and protein expression profile of ASCs and BMSCs are extremely rare. However, an important comparative micro-array analysis of mesenchymal stem cells obtained from bone marrow, adipose tissue, and umbilical cord clearly showed that no significant differences in morphology, immune phenotype, and differentiation capacity were apparent between the groups (29). When compared to fibroblasts, 25 genes overlapped and were up-regulated in all cellular preparations (29). No phenotypic differences could be determined comparing 22 surface antigens. Interestingly, hundreds of expressed sequence tags that were differentially expressed between groups. There is some controversy in the literature regarding the expression of the widely used marker of hematopoietic stem cells, CD34, in ASCs (30-32). Data suggest that CD34 is absent in cultured ASCs but, by contrast, higher levels of expression especially early in passage or lower subculture number have been documented (32). The Stro-1 antigen, a typical BMSC surface antigen, has also been reported to have controversial expression levels as it has been described to be absent and present
in human ASCs (13, 33). The conflicting data might be due to the differences in either the epitope recognized, the duration in culture, hematopoietic cell contamination, or the detection methods, typically flow cytometry or immunohistochemistry. While both cell types share numerous surface markers, there are some differences in protein expression between the groups as seen when comparing ASCs and BMSCs taken from the same individual. BMSCs express the marker CD106 while ASCs do not, but conversely ASCs express CD49d unlike bone marrow cells (33).

The expression of important transcription factors in cultured ASCs is still present even after 30 passages. The mRNA expression of Nanog, Oct-4, and Sox-2 was determined at passage 30 with the expression levels of all genes being significantly lower than passage 4, yet still detectable (24). These factors have been shown to play an important role in differentiation of ASCs (24). Furthermore, gene expression is also affected by the time spent in culture between passages. Culturing of cells every 14 days instead of the typical 5 days resulted in increased expression of the stem cell-related genes thus suggesting that these cells might have a stronger differentiation potential. Therefore, the ideal cells for therapeutic transplantation would be of a low passage with a longer time spent in between passages.

It is important for transplanted cells not to induce an immune response from the host. Like cells isolated from bone marrow, ASCs do not provoke in vitro alloreactivity of incompatible lymphocytes, and they suppress mixed lymphocyte reaction and lymphocyte proliferative response to mitogens in a dose and time dependent manner (34). This is most likely due to the fact that less than 1% of ASCs express the HLA-DR protein, which is known to mediate the rejection of transplanted tissue in the graft-versus-host immune response (35).
Moreover, Rodriguez et al (35) demonstrated that adipose derived cells are immunoprivileged in vitro and in vivo being relatively resistant to rejection after transplantation. These findings support the idea that ASCs share immunosuppressive properties with BMSCs and therefore might represent an alternative cellular source suitable for allogenic transplantation procedures lacking the risk of tissue rejection.

**Differentiation Capacity**

In order for ASC transplantation to function properly and repair the damaged myocardium, ASCs must have the capability to differentiate into cardiac cells thus being able to repopulate cellular loss after infarction due to ischemia. Fortunately, differentiation of ASCs into cells that phenotypically resemble myocytes has been detailed in several in vitro experiments (36-38). Lineage specific differentiation of stem cells can be controlled by chemical treatment, co-incubation with other cells, or adding growth factors to the culture medium. It has been demonstrated that treatment with the commonly used DNA demethylating agent, 5-azacytidine (9 μmol/L for 24 hours), results in differentiation of ASCs isolated from New Zealand White rabbits into myocytes after 3 weeks in culture. The differentiated cells were multinucleated, began to beat spontaneously in culture and positively expressed myosin heavy chain, α-actinin, and Troponin-I (39).

Besides chemical treatment, addition of growth factors to the culture medium will also influence the differentiation pathway. It has been documented that medium supplemented with interleukin-3,-6, and stem cell factor induces differentiation of ASCs taken from male C57Bl/6N mice into myocytes that also have pacemaker activity (15). After 24 days in culture,
cells were beating in unison independently of 5-azacytidine treatment and clones expressed several cardiac-specific mRNA such as GATA-4, Nkx2.5, ventricular and atrial myosin light chains. Clones were also positive for the cardiac markers: myosin-enhancing factor 2C, α-actinin, myosin heavy chain and connexin 43, while being negative for skeletal muscle markers. Structural analysis by electron microscopy revealed multinuclear cells with morphology consistent with cardiac myocytes. Cellular electrical activity was recorded on cells in a current clamp and revealed an action potential characteristic to cardiac pacemaker cells. The differentiated cells were also capable of responding to both adrenergic and cholinergic agonists. As expected for myocyte-like cells, isoproterenol, a β-agonist, induced a dose-dependent increase of the spontaneous contraction rate while propranolol, a nonselective β-adrenergic antagonist, reversed the effects. In contrast, the nonselective acetylcholine agonist, carbamylcholine stopped the spontaneous contractions. Furthermore, reversibly permeabilized human ASCs co-incubated with nuclear and cytoplasmic extracts from neonatal rat myocytes resulted in differentiation into binucleated striated spontaneously beating myocytes (39). Finally, it has been reported by Song et al (41), using standard culture conditions without any addition of growth factors or cytokines, that ASCs isolated from humans can spontaneously differentiate into myocytes after 12 days in culture. Vascular endothelial growth factor (VEGF) was identified as being critical for cardiomyogenesis (41). In fact, significant amounts of VEGF were found in the conditioned medium which is known to significantly enhance MHC-α, cTN-I, and Nkx2.5 expression in differentiated embryonic stem cells suggesting that VEGF is partly responsible for the differentiation into cardiac cells via a paracrine mechanism. It has been clearly demonstrated that ASCs will differentiate into cardiac myocytes in vitro, but a limited
number of studies have detailed differentiation in an *in vivo* animal model of myocardial infarction. Injection of ASCs taken from β-galactosidase transgene expressing Rosa26 mice into B61295 mice immediately after permanent occlusion resulted in β-galactosidase positive cells expressing the cardiac specific genes, myosin heavy chain, Nkx2.5, and Tropinin I (26). Similar results were obtained when using green fluorescent protein (GFP) labeled ASCs in a rat model of heart failure in which cells were immunohistochemically stained positive for cardiac markers 30 days post surgery (40). Moreover, it has been reported that ASCs grown in temperature responsive culture dishes formed a monolayer sheet due to cell-to-cell adhesions that, when transplanted onto the ligated myocardium 4 weeks post surgery, resulted in a thickened layer of newly generated vessels and myocytes over the damaged area that were positive for Tropinin I and desmin (41). These data strongly suggest that there is a great potential for ASC cellular therapy as a treatment to repopulate the injured myocardium with differentiated cells that have a functional pacemaker activity while phenotypically and structurally resemble myocytes.

Therapeutic enhancement of neovascularization is an important strategy needed to limit the complications of post ischemic injury. Stem cell therapy has been shown to be promising in neoangiogenesis in models of hind-leg ischemia (19, 42). The ability to differentiate into mature endothelial cells, which is critical for formation of new blood vessels, gives ASCs great angiogenic potential. Planat-Benard et al (43) observed vascular-like structure formation in Matrigel plug using ASCs taken from mice. Cells were positive for the endothelial markers: CD31, VE-cadherin, and von Willebrand factor. Moreover, the cells formed branching networks, consistent with the formation of vascular structures that lead to enhancement of the neovascularization reaction in ischemic tissue. It has also been shown that ASCs can form
numerous tube-like structures, and, while in the presence of erythrocytes, demonstrated the existence of a functional vascular structure (44). Similarly, CD31 expression and differentiation into vascular structures is enhanced by VEGF. If VEGF were added to the growth medium, human ASCs display an endothelial phenotype (45). All these results clearly demonstrate that there is a relationship between VEGF and lineage specific differentiation. In vivo studies also support the potential of endothelial differentiation. Using a model of hind-limb ischemia in rats, transplantation of ASCs improves angiogenesis and recovery of vascular blood supply (19). Several groups have reported similar results that transplanted ASCs can integrate as fully functional and differentiated endothelial cells (26, 46-48). Furthermore, Zhang et al (48) demonstrated that BrdU-labeled ASCs differentiate into myocytes and endothelial cells that participate in vessel-like structure formation. It is clear that cellular transplantation of stem cells derived from adipose tissue is a viable option for repairing damaged myocardium through differentiation into myocytes and endothelial cells that become an integral part of new vascular structures.

**Potential for Myocardial Regeneration**

Over the past several years, experimental findings suggest there is a therapeutic potential for cellular replacement therapy as treatment of MI and other progressive chronic cardiac diseases such as left ventricular (LV) remodeling and heart failure. Since cardiovascular disease remains a worldwide problem, the development of novel effective cell-based therapies is crucial to improve patient outcome post MI. Current treatment of MI still leaves a significant number of patients with impaired cardiac function that leads to more severe LV dysfunction and adverse
remodeling. Remodeling of the ventricle is a result of increased apoptosis in the ischemic zones after infarction. While apoptosis influences remodeling, the other form of cellular death that occurs in the heart, necrosis, provokes inflammatory reactions, neoangiogenesis, fibroblast activation, and scar formation. To date, most of the cellular based therapies have involved the use of myoblasts or BMSCs that often result in an improvement in LV function but have little effect on preventing LV remodeling (22, 49, 50). Recently, studies have shown that ASCs have become a viable alternative option to further limit remodeling and the progression to LV dysfunction post MI (29, 47, 48, 51, 52). In a model of acute MI, rats underwent ligation of the left anterior descending coronary artery for 45 minutes, then were allowed to reperfuse for 15 minutes before receiving an intramyocardial injection of GFP-labeled ASCs. Interestingly, 12 weeks later, there was very poor engraftment, but cell-treated animals had more capillaries and arterioles per mm$^2$ in the infarct border zone with a similar trend in the infarct area. The remodeling seen in the control animals was not detected in the ASC-treated group (51). Furthermore, a significant increase in LV ejection fraction and fractional shortening compared to control mice at 2 weeks following permanent occlusion of the coronary artery has been reported. The improvement in cardiac function also correlated with a significant decrease in LV end-systolic diameter (53). Interestingly, reversed wall thinning in the scar area has been seen at 30 days after permanent ligation (40). The reconstruction of thick myocardial tissue reduces the stress on the LV wall subsequently improving cardiac function. Similar results from a direct comparison of intracoronary injection of ASCs and BMSCs in a porcine model of MI revealed that ASC treatment substantially improved LV perfusion, function and attenuated adverse remodeling. The capillary vessel density was found to be greater in the ASC treatment group
versus the group receiving BMSCs (40). This data suggest that angiogenesis may contribute to the maintenance of cardiac function by preservation of the remaining viable myocytes and through neovascularization, thus protecting the myocardium in the border zone that would normally undergo apoptosis.

**Mechanisms of Action**

There are three proposed mechanisms through which ASCs can be used to repair and regenerate damaged myocardium: myocyte regeneration, vasculogenesis, and paracrine actions. Cell therapy as treatment of cardiovascular diseases was originally thought to repopulate the myocardium, but growing evidence supports the hypothesis that paracrine mechanisms play an essential role in repairing the damaged myocardium. Paracrine factors are released from endogenous cells of the heart in response to injury. These pro-survival growth factors or cytokines mediate multiple mechanisms such as increased blood flow to ischemic tissue, reduction in myocyte apoptosis, regulation of inflammatory response, and recruiting endogenous stem cells to regenerate injured tissue. Additionally, administration of conditioned media from hypoxic ASCs significantly increased endothelial cell growth and reduced endothelial cell apoptosis, while transplantation of these cells into ischemic hind limbs led to improved perfusion, suggesting that paracrine factors from these cells promote neovascularization (54). Similar effects have been seen in the heart as injection of conditioned media decreased apoptosis, fibrosis, and LV dilatation and increased myocardial thickness after infarction (48). The absence of cells in the treatment proves that a paracrine mechanism from growth factors released in the conditioned media plays an important beneficial role in repairing the damaged myocardium.
Induction of neovascularization is crucial for limiting the damage in ischemic tissue; however, studies suggest that only a small number of blood vessels contain transplanted donor cells. It is known that angiogenesis and arteriogenesis typically involve mediators such as nitric oxide, VEGF, basic fibroblast growth factor (b-FGF), hepatocyte growth factor (HGF), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and the angiopoietins. Interestingly, hypoxic preconditioning of cells induces expression of activated Akt and endothelial nitric oxide synthase (eNOS), while also secreting higher levels of VEGF, b-FGF, HGF, insulin-like growth factor (IGF)-1 when compared to cells cultured under normal culture conditions. The transplantation of the cells led to a significant increase in blood vessel density (55). The release of the angiogenic factors augmented the vessel number without incorporation into mature vessels. Tissue levels of VEGF and b-FGF are significantly increased in infarcted hearts that are treated with ASCs (56). Moreover, the expression of these growth factors correlated with increased angiogenesis and reduction in infarct size.

Besides the increase in new blood vessel formation, it is important that the paracrine factors function to protect resident cells, particularly myocytes, from apoptosis. Transplantation of ASCs along with their secretion of VEGF, b-FGF, IGF, and SDF-1 has been shown to upregulate the anti-apoptotic protein, Bcl-2, which results in the decrease in myocyte apoptosis in vitro and in vivo (57). Furthermore, intramyocardial injection of adenoviruses over-expressing VEGF or b-FGF decreases infarct size and increases expression of Bcl-2 (21). It has been shown that stem cells over-expressing Akt led to a decline in myocyte apoptosis in vitro and that transplantation led to a decrease in infarct size (57, 59). The Akt over-expressing cells secreted several paracrine factors such as VEGF, b-FGF, thymosin β4 (TB4) and HGF. HGF has been
shown to have anti-apoptotic effects in acute MI (60). Additionally, transplantation of ASCs over-expressing HGF into MI rat models induced myocardial angiogenesis, suppressed fibrosis and improved cardiac function better than transplantation of ASCs alone (61). Also the G-actin sequestering peptide, TB4, promotes survival of embryonic and postnatal cardiac myocytes, and treatment results in enhanced myocardial survival suggesting that ASCs protect the myocardium from apoptosis through paracrine effects (62). Myocardial injury provokes an inflammatory response resulting in an increased expression of a variety of both pro-inflammatory and anti-inflammatory cytokines. Initially, inflammatory cytokine expression is necessary for maintaining homeostasis in the heart after stress or injury, however, sustained upregulation of certain cytokines leads to adverse remodeling and heart failure (63). There is increasing evidence that ASCs secrete cytokines that may directly act to limit deleterious, sustained endogenous inflammation. In fact, cellular administration led to a downregulation of TNF-α, IL-1β, and IL-6, which are involved in adverse LV remodeling (64). Furthermore, cell transplantation attenuated myocardial dysfunction in a rat model of acute myocarditis (65). Also, conditioned media protected isolated adult rat cardiac myocytes from MCP-1 induced injury, suggesting that the anti-inflammatory effects were due to paracrine factors. Data also suggests that ASCs may directly modulate T lymphocyte function in the heart, possibly leading to protection against their cytotoxicity or alternatively modulate their role in cardiac remodeling. T lymphocytes co-cultured with cardiac fibroblasts led to an increase in fibroblast pro-collagen expression, suggesting that suppression of T lymphocyte accumulation may inhibit fibrosis (66). Therefore, alterations in the immune response by cellular therapy may serve to improve LV function and attenuate adverse LV remodeling.
The direct effect on fibrosis by stem cell therapy has been demonstrated. Conditioned media significantly attenuated proliferation of cardiac fibroblasts and up-regulated elastin, myocardin, and DNA-damage inducible transcript 3 (67). Furthermore, type I and III collagen expression and type III collagen promoter activity were significantly down-regulated. Gene expression analysis revealed that stem cells had several matrix-modulating factors up-regulated such as matrix metalloproteinase-2 (MMP-2), tissue inhibitors of matrix metalloproteinases (TIMP)-1 and TIMP-2, thrombospondin-1, and tenacin C, suggesting a direct effect on extracellular matrix remodeling (68). A reduction in fibrosis, along with a reduction in levels of MMP-2 and MMP-9, has been documented after injection of ASCs in models of MI (68). IL-1β, which is secreted by ASCs, has a direct anti-proliferative effect on cardiac fibroblasts (20). The paracrine factors secreted by ASCs may play a crucial role in extracellular matrix remodeling that contributes to improvements in LV function. Figure 1 depicts the proposed mechanism of how ASC transplantation mediates cardioprotection through differentiation, neovascularization, and paracrine effects on the host cells.
Figure 1. Proposed mechanism(s) of ASC transplantation in mediating cardioprotection. Addition of growth factors to the medium, preconditioning with hypoxia or adenoviral overexpression of Akt or HGF enhances differentiation into cardiomyocytes or endothelial cells while also inducing a greater survival paracrine effect in host cells.
Preconditioning of Stem Cells

Various animal studies show the potential to regenerate myocardium, improve perfusion to the infarct area, and improve cardiac function (69-72). Phase II and III clinical studies indicate that stem cell transplantation is feasible and may have beneficial effects on ventricular remodeling after myocardial infarction. However, the majority of transplanted cells are readily lost after transplantation because of the poor blood supply, ischemia/reperfusion injury, and inflammatory factors. Disconcerting reports have shown that up to 99% of transplanted cells are lost within the first 24 hours (73). Therefore, enhancing cell viability and reduction of apoptosis of ASCs in an ischemic microenvironment of the infarcted heart is critical for improving the efficiency of cell therapy. To improve the effectiveness of stem cell transplantation various methods have been employed to increase cell survival. Recently, Zhang et al. demonstrated that ischemic preconditioning of stem cells attenuated apoptosis induced by simulated ischemia (SI) and re-oxygenation (RO) (74). Subjecting cells to sublethal bouts of hypoxia prior to SI/RO resulted in preconditioning that correlated with stabilized membrane potential, upregulation of Bcl-2 and VEGF. Furthermore, there was an increased phosphorylation of ERK and Akt (74). Other models have shown that the effect of ischemic preconditioning can be mimicked pharmacologically by using phosphodiesterase-5 (PDE-5) inhibitor, sildenafil (75). Using mitoK$_{ATP}$ channel opener, diazoxide, Ashraf et al. preconditioned skeletal myoblasts to promote their survival in the infarcted heart (76). Diazoxide preconditioning of the cells significantly induced expression of p-Akt, b-FGF, HGF, and COX-2. Treatment of cells with wortmannin prior to preconditioning abolished the effects and significantly reduced their survival thus
demonstrating the importance of the PI3K-Akt signaling cascade. Positive results have been demonstrated using genetic modulation of stem cells with transgenes that overexpress angiogenic growth factors such as Ang-1, VEGF, or Akt to improve cell survival, neovascularization, and cardiac function by limiting the remodeling process in the scar while decreasing apoptosis of myocytes in the peri-infarct region (77, 78). Moreover, genetically modified cells seem to function in autocrine and paracrine manner to confer therapeutic effects. Transplantation of cells over-expressing VEGF not only were protected from apoptosis but also reduced the apoptotic index of host myocytes. There was also improvement in regional blood flow in the myocardium leading to preservation of cells and myocardial structure. Jian et al. (77) showed the cytoprotective effects of co-overexpression of Ang-1 and Akt. Transduction with Ang-1 and Akt genes resulted in marked survival of the transplanted cells in vivo, their differentiation into myocytes and participation in neovascularization, which caused a reduced infarct size and optimally preserved cardiac function after MI (77).

**PDE-5 Inhibitors**

Phosphodiesterase type-5 (PDE-5) inhibitors are a class of vasoactive drugs that have been extensively used for treatment of heart failure, pulmonary hypertension, and coronary artery disease besides their use for the treatment of erectile dysfunction (79-82). The mechanism of action involves active inhibition of the PDE-5 enzyme resulting increase in cyclic guanosine monophosphate (cGMP) and smooth muscle relaxation in the penis. There are 11 families of PDEs that have been identified in mammalian tissues (83, 84). The PDEs vary in their substrate specificity for cyclic adenosine monophosphate (cAMP) and cGMP: PDE-5, PDE-6 and PDE-9.
are specific for cGMP; PDE-4, PDE-7 and PDE-8 are specific for cAMP; and PDE-1, PDE-2, PDE-3, PDE-10 and PDE-11 have mixed specificity for cAMP/cGMP (85). PDE-5 inhibitors compete with the substrate cGMP for binding to the protein at the catalytic site. Although cGMP binding to the catalytic site stimulates cyclic-nucleotide binding to the allosteric sites, inhibitors do not elicit the same property, and Ser92 phosphorylation has no effect on inhibitor binding.

PDE-5 is the primary enzyme with cGMP-hydrolyzing activity in human corpus cavernosal tissue (85). PDE-5 inhibitors have been studied extensively for their role in regulation of vascular tone and blood-flow balance during erection. During erection, nitric oxide (NO) is released from non-cholinergic, non-adrenergic neurons and from endothelial cells. NO diffuses into cells, where it activates soluble guanylyl cyclase, the enzyme that converts GTP to cGMP, which then stimulates protein kinase G (PKG) and initiates a protein phosphorylation cascade. This results in a decrease in intracellular levels of Ca2+ ions, leading to dilation of the arteries that bring blood to the penis and compression of the corpus cavernosum. A PDE-5 inhibitor inhibits enzymatic hydrolysis of cGMP by binding to the cGMP-catalytic sites thereby allowing the accumulation of cGMP in the erectile tissue (85).

PDEs are found in all tissues besides the human corpus cavernosal tissue, but the distribution of the PDEs varies among different tissues and cell types (86). PDE-5A has a wide distribution in the body tissues and cells where it exists in three isoforms; PDE-5A1, PDE-5A2, PDE-5A3 that only differ in their N-terminal sequence (87). Immunoistochemical studies have demonstrated the presence of PDE-5A isoforms in vascular and bronchial smooth muscle and in platelets. It is not clear whether PDE-5A is present in the human myocytes. However, a recent study by Senzaki et al. (88) provided evidence for PDE-5A expression in canine cardiomyocytes.
and our laboratory reported expression in mouse heart (75). Despite these interesting observations, little is known about the distribution of PDE-5A in stem and progenitor cells. However, recently it has been reported that cultured bone marrow derived mesenchymal stem cells predominantly express PDE-5A (89).

The cytoprotective effects of PDE-5 inhibitors have been observed in heart cells, neurons and glia, and epithelial cells. The precise mechanisms for these protective effects are quite complex. We have demonstrated that sildenafil (Viagra®) and other PDE-5 inhibitors induce powerful protective effect against ischemia/reperfusion injury in the rabbit and mouse heart (90-94), DOX-induced cardiomyopathy (95, 96) and myocardial infarction-induced heart failure in mice (97). Furthermore, we showed that PDE-5 inhibition protects isolated adult cardiomyocytes from SI/RO. The cardioprotective effect is attributed to limiting apoptosis and necrosis through enhanced expression of nitric oxide synthases (NOS), particularly, eNOS/iNOS, activation of protein kinase C and PKG, phosphorylation of ERK1/2, PKG-dependent phosphorylation of GSK-3β, NO-dependent upregulation of Bcl-2/Bax and opening of the mitochondrial K_{ATP} channels (75, 91-93, 97, 98).

**Nitric Oxide/cGMP/Protein Kinase G Signaling**

Nitric Oxide (NO) is well recognized as a key mediator in cell signaling processes. It is produced from L-arginine through chemical reaction catalyzed by at least three major isoforms of nitric oxide synthase, i.e. neuronal (nNOS), inducible (iNOS), endothelial (eNOS). NO/cGMP/PKG signaling is a widely studied pathway in many tissues and cells, and reduced production and function of NO has been shown to participate in a number of disorders such as
cardiovascular, pulmonary, endothelial, renal, and hepatic diseases and erectile dysfunction. NO is produced and released from many cell types in the body where it acts as a paracrine signal in a number of systems, including the vasculature. NO at nanomolar levels binds tightly to a prosthetic heme on the β-subunit of NO-GC, also known as the soluble guanylyl cyclase, and causes activation of the enzyme (99, 100). Activation of NO-GC increases conversion of GTP to cGMP, resulting in elevation of cGMP, which initiates the cGMP-signaling pathway and subsequent physiological changes (101). NO-induced elevation of cGMP regulates numerous physiological processes including: relaxation of vascular smooth muscle, inhibition of platelet aggregation, inhibition of cytokine production, blunting of cardiac hypertrophy, and protection against myocardial ischemia/reperfusion injury (102-104). At the cellular level NO and cGMP regulate important processes such as growth, survival, differentiation, proliferation, and migration. These effects are largely mediated through activation of cGMP dependent protein kinase I, PKG. (105-108). cGMP-gated channels, PDEs and PKGII are also important targets for cGMP actions.

PKG is a serine/threonine protein kinase and is one of the major intracellular receptors for cGMP. PKG is present in high concentrations in smooth muscle, platelets, cerebellum, hippocampus, dorsal root ganglia, neuromuscular end plate, and the kidney vasculature (109). PKGI isozymes (PKGIα and PKGIβ) are products of alternative splicing and differ only in the N-terminal amino acids (110, 111). PKG Iα is more sensitive to activation by cGMP. The activation of PKG phosphorylates many intracellular proteins and regulates important physiological functions such as relaxation of vascular smooth muscle, inhibition of cell differentiation and proliferation, and inhibition of platelet aggregation and apoptosis (109, 112). PKG also plays an
important role in the cardiovascular system. Recently, we reported that sildenafil-induced protection is dependent upon activation of PKG in adult mouse heart and cardiomyocytes (113). This notion is based on the fact that PKG inhibitors and selective knockdown of PKG by adenoviral vector containing short-hairpin RNA of PKG abolished the antinecrotic and antiapoptotic effect of sildenafil in cardiomyocytes, and PKG inhibition also abrogated the infarct size reduction by sildenafil in isolated mouse hearts. Furthermore, our laboratory also has shown the effect of PKG Iα overexpression in protecting cardiomyocytes from SI/RO induced necrosis and apoptosis (114).

Although research on stem cells has increased dramatically in recent years, there are very few studies on the role of the NO/cGMP/PKG pathway in stem cells (115-121). It has been reported that the NO/cGMP/PKG signaling pathway has been proposed to promote stem cell-like characteristics in glioma cells in the tumor perivascular niche of medulloglioma (118). Interestingly, enhanced endogenous NO generation has been shown to positively regulate proliferation of neural progenitor cells through activation of cGMP/PKG signaling pathway (119). Recent studies have suggested the role cGMP-mediated NO signaling plays in the differentiation of embryonic stem cells into myocardial cells (120, 121). Furthermore, expressions of mRNA and protein levels of the three NOSs and sGC, along with expression of PKG have been shown to increase during differentiation (116). However, the role of NO/cGMP/PKG signaling in protection of ASCs against ischemia has not been investigated.
Rationale for Study:

The present study, a series of novel investigations designed to examine the feasibility of effect of PDE-5 inhibition as a strategy to precondition human ASCs for improving their efficacy in vivo after cardiac transplantation. The rationale for this approach was the established powerful preconditioning-like effect of PDE-5 inhibitors in cardiomyocytes (75, 98, 114) and against ischemia/reperfusion injury in heart (92-94) previously established by our laboratory. The purpose of the following study is to investigate the effect of PDE-5 inhibition of ASCs for transplantation to attenuate adverse cardiac remodeling and preserve of cardiac function in a chronic model of MI. Furthermore, we wanted to attain a better understanding of the signaling pathways involved, which ultimately lead to enhancement of stem cell therapy.

Accordingly, the main aims of the present study were:

Aim #1) to investigate whether PDE-5 inhibition could confer cytoprotection of ASCs against SI/RO injury in vitro;

Aim #2) to demonstrate the role of cGMP-dependent PKG signaling in protection of ASCs;

Aim #3) to show that in vivo transplantation of ASCs after ex vivo PDE-5 inhibition improve LV function following myocardial infarction;

Aim #4) to examine the possible role of paracrine mechanism in enhancing the cytoprotective effects of PDE-5 inhibition.
ABSTRACT
Several studies have implicated cellular replacement therapy as a treatment strategy for myocardial infarction, although the results have been limited. Therefore, enhancing the therapeutic potential of stem cells injected into ischemic microenvironments by novel preconditioning strategies is critical for improving cellular therapy. We tested the hypothesis that inhibition of PDE-5 with sildenafil (Viagra®) or selective knockdown with a silencing vector in adipose derived stem cells (ASCs) would improve their survival after simulated ischemia/reoxygenation (SI/RO). ASCs were treated with sildenafil or infected with a PDE-5 silencing vector shRNA (shRNA\textsuperscript{PDE-5}) and subjected to SI/RO. Both sildenafil and shRNA\textsuperscript{PDE-5} significantly reduced cell injury with improved viability, decreased lactate dehydrogenase release and reduced apoptosis. The preconditioned ASCs demonstrated an increase in the release of nitric oxide metabolites and growth factors including VEGF, b-FGF, and IGF into conditioned medium in which treatment protected adult cardiomyocytes from SI/RO. The cytoprotective effect seen in ASCs against SI/RO injury was abolished by inhibition of protein kinase G (PKG) with a pharmacological inhibitor and selective knockdown with shRNA\textsuperscript{PKG}. Our data shows that \textit{in vitro} inhibition of PDE-5 using either genetic or pharmacological approaches can improve stem cell therapy following myocardial infarction.
Introduction

The main factor leading to the progression of heart failure is the irreversible loss of cardiomyocytes due to necrosis and apoptosis following ischemic injury. To overcome myocyte loss and the heart’s limited self-regeneration capacity, recent research has focused on transplantation of stem cells to differentiate and replenish the loss of myocytes. Various animal studies have shown the potential to regenerate myocardium, improve perfusion to the infarct area, and improve cardiac function (69-72). Although cardiac performance by cell-based therapy has improved, unsatisfactory cell retention and transplant survival still plague this technique. The available transplantation strategies achieve modest engraftment of donor stem cells in the infarcted myocardium, primarily due to the rapid and massive loss of donor stem cells (122, 123). Several factors influence the accelerated cell death in the infarcted myocardium, including the ischemic and cytokine-rich microenvironment, mechanical injury, maladaptation, and the origin and quality of the donor cell preparation (124). Therefore, strategies targeted toward enhancing stem cell survival in the ischemic microenvironment are of paramount importance for improving cardiac regeneration. Previous studies have shown that treatment of bone marrow stem cells (BMSCs) with hypoxia improved survival post engraftment in the infarcted heart (125), increased proliferation rates and differentiation, and modulated their paracrine activity (126). In addition, various pharmacological preconditioning agents including diazoxide, an opener of mitochondrial K_{ATP} channel (77), vascular endothelial growth factor 2 (127), and IGF-1 (128) have been shown to promote myogenic response of stem cells following transplantation in the myocardium. Nevertheless, progressive strategies to improve the regenerative potential of stem cells are critical for their utility.
In the present study, we tested the hypothesis whether PDE-5 inhibition could improve the survival of adipose derived stem cells (ASCs,) leading to enhanced cardiac function following myocardial infarction in mice. Specifically we addressed the following questions: 1) Does PDE-5 inhibition by sildenafil or genetic knock-down with a silencing vector improve survival following simulated ischemia/reoxygenation (SI/RO) injury \textit{in vitro}?. 2) What is the role of cGMP-dependent PKG signaling pathway in protection of ASCs?; 3) What is the role of paracrine mechanisms for enhancing cytoprotective effects of PDE-5 inhibition? Our results show that preconditioning of ASCs enhances release of cytokines such as VEGF, b-FGF, and IGF-1, stimulates NO metabolites, activates PKG, and increases cell viability after SI/RO, all of which were abolished with PKG inhibition.
Materials and Methods

Isolation of Adipose Derived Stem Cells

Epicardial adipose tissue was harvested from voluntary patients undergoing transplant. Adipose tissue was mechanically disrupted with a scalpel and washed twice with phosphate buffered saline. Minced fat tissue was digested for an incubation period of 90 min at 37°C on a shaker in 20 mL of sterile filtered PBS containing 25 mg of Collagenase type VIII (Sigma-Aldrich) and 5 mM calcium chloride. The digested tissue was filtered through a 100 μm nylon mesh filter (Millipore). Filtrate was centrifuged at 800 x g for 10 min. The supernatant containing adipocytes and debris was discarded and the pelleted cells were washed twice with 40 mL Hank’s Balanced Salt Solution (Cellgro). Freshly isolated ASCs were plated with α-MEM containing 20% FBS, 1% L-glutamine (0.2 M, Cellgro) and 1% Penicillin (10,000 U/mL) with Streptomycin (10 mg/mL, Cellgro). Plastic adherent cells were named human adipose tissue derived stem cells (ASCs) and were grown in culture at 37°C in a humidified incubator at 5% CO₂ followed by daily washing for three days to remove red blood cells and non-adherent hematopoietic cells. ASCs were then plated after 3 days for subsequent experiments at a density of 1000 cells/cm². Subsequent passages were performed with a 0.25% trypsin solution containing 0.01% EDTA for 6 minutes at 37°C.

Flow Cytometry

Cell surface antigen phenotyping was performed on ASCs that were harvested upon reaching 90% confluency. Cells were pelleted at 500 x g for 5 minutes at 4°C. Approximately,
1 x 10^6 ASCs were incubated for 1 h at 37°C with primary FITC conjugated antibodies at a dilution of 1:1000 for CD 14, CD29, CD44, CD45, CD105 and HLA-DR2 (Invitrogen, Molecular Probes.) Mild agitation was used every 10 min to further mix the solution and prevent cell clumping. No antibody controls were tested for each individual antibody. Cells were counted on a Beckman CoulterElite XL-MCL single-laser flow cytometer at a minimum of 10,000 counts. Positive results were defined as over 97% of cells expressing the surface protein of interest.

**Differentiation of ASCs**

Adipogenesis was induced in ASCs using a mesenchymal stem cell adipogenesis kit (Millipore) according to manufacturer’s instructions. In brief, ASCs were plated at a density of 60,000 cells per well in a 24-well culture dish in 1 mL of medium and incubated at 37°C in a 5% CO₂ humidified incubator overnight. Once reaching 100% confluence, medium was aspirated and 1 mL of adipogenesis induction medium (low glucose DMEM containing 10% FBS, 1 μM dexamethasone, 0.5 mM IBMX, insulin (10 μg/mL), 100 μM Indomethacin, and 1 % Pen/Strep) was added. This medium change corresponds to differentiation day 1. Adipogenesis induction medium was changed every 2-3 days for 21 days. Positive staining of Oil Red-O indicated adipogenic phenotype.

Osteogenesis was induced in ASCs using HyClone AdvanceSTEM Osteogenic Differentiation Kit (Thermo Scientific) according to manufacturer’s instructions. In brief, ASCs were plated at a density of 60,000 cells per well in a 24 well culture dish with 1 mL of medium and incubated at 37°C in a 5% CO₂ humidified incubator overnight. Once reaching 100%
confluence, medium was aspirated and 1 mL of osteogenic induction medium supplemented with 1 nM dexamethasone, 2 mM β-glycerophosphate, and 50 μM ascorbate-2-phosphate. ASCs were induced for 14 days and the osteogenic medium was replaced every 2-3 days. Osteogenic mineralization was assessed after 21 days by staining with 40 mM Alizarin red (Sigma).

**Immunocytochemistry**

ASCs were cultured on sterile glass cover slips and fixed by incubation in 4% paraformaldehyde/ PBS for 20 min and permeabilized with 1.0% Triton X-100 in PBS for 10 min. Intracellular staining patterns and distribution of Oct-4, Sox-2, Nanog, and PDE-5 proteins were analyzed by immunostaining with incubation of respective antibodies at 4°C overnight (1:500 dilution) followed by incubation of FITC conjugated secondary antibodies at 37°C for 1 h (1:1000 dilution). Staining of 4’,6-diamino-2-phenylindole (DAPI; Sigma) was used to visualize all nuclei.

**Western blot analysis**

Total soluble protein was extracted from ASCs with extraction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 50 μg/ml PMSF, protease inhibitor cocktail (10μl/ml, Sigma), 0.3% β-mercaptoethanol as described by Qiu et al. (129). Homogenate was centrifuged at 14,000 x g for 10 min at 4 °C, and the supernatant was recovered as the total cellular protein. Total protein (50 μg) from each sample was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and then blocked with 5% nonfat dry milk in Tris-
buffered saline. The membrane was subsequently incubated with a primary antibody at a dilution of 1:500 for each of the respective proteins, *i.e.* PDE-5A, Oct-4 (rabbit polyclonal), PKG, β-actin (goat polyclonal, Santa Cruz), SOX-2, and Nanog (mouse monoclonal, BD Biosciences). The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution, 1 h at room temperature). The membranes were developed using enhanced chemiluminescence system (ECL Plus; Amersham Biosciences) and exposed to X-ray film.

**Preparation of shRNAs**

PDE-5 gene silencing shRNA (inserted into miRNA-155 cassette) and gfp-PDE-5 fusion protein both coupled to a CMV promoter and incorporated into adenoviral vectors were generated by Zhang et al (130). In brief, short-hairpin RNAs were designed based on mouse PDE5A sequence

(shRNA\textsuperscript{PDE5-1899}: FORWARD 5′-TGCTGTTCAGAGCAGCAAACATGCAGTTTTGGCCACTGACTGACATCTGC TCTGAAA-3′ and REVERSE 5′-CCTGTTTCAGAGCAGCAAATCGACTGACATCGCATGTTCATGC CTGAAAC-3′; shRNA\textsuperscript{PDE5-2066} FORWARD 5′-TGCTGAAATGTATGGTGTTCATGATGGTTTTGGCCACTGACTGACCATCATGGCACC ATCATTT-3′ and REVERSE 5′-CCTGAAATGTATGGTGCCATGATGGTCAGTCAGTGCGCCACTGACCACCATCATGCGAA-3′)
and inserted into pcDNA 6.2-GW/EmGFP-miR-155 vector (Invitrogen) retaining miRNA regulatory sequences required for efficient shRNA processing. This was transferred by recombinase cloning into pAd/CMV/V5-DEST™ vector (Invitrogen) to generate AdV-gfp-shRNA_{PDE5A}. AdV was CsCl purified and titered at 1.5–3.0 × 10^{10} pfu/ml. Green fluorescent protein (gfp) enabled infection to be confirmed (AdV-gfp virus was the control), and the miRNA construct allowed use of the CMV promotor enhancing gene knock-down.

The mouse PKGI, shRNA expression vector for PKG was constructed as described previously (98). In brief, to knockdown the expression of PKG, we used shRNA, targeting the mouse cDNA of PKG type I (GenBank™ accession number NM_001013833): corresponding to bases 1593 to 1611, targeting the sequence 5′-GAACAAAGGCCATGACATT-3′, synthesized by Dharmacon Research Inc. (Lafayette, CO). A non-targeting scrambled RNA duplex siRNA control (NTSC, Dharmacon) containing 21-nucleotide sequences demonstrating no homology to murine genes was also used as a control for transfection. Transient transfections of duplex siRNAs (100 nM) were performed in H9C2 cells using siPORT™ amine (Ambion). After 48 h, RNA was isolated by Tri-Reagent (Molecular Research Center), and RT-PCR and quantitative real-time PCR were performed. After confirming the significant reduction of PKG expression in H9C2 cells using this duplex siRNA targeting the mouse PKGI, shRNA expression vector for PKG was constructed using the pSilencer™ adeno1.0-CMV system from Ambion (Adenoviral siRNA expression Vector System). The hairpin siRNA oligonucleotide (55-mer) sequence 5′-TCGAGGAAACAAAGGCCATGACATTttcaagagaAATGTCATGGCCTTTGTTCAGA-3′ (mouse PKGI with sequence in capital letters and loop in lowercase italics) and its antisense with
XhoI and SpeI were synthesized, annealed, and subcloned into the pSilencer adeno 1.0-CMV shuttle vector. HEK293 cells were transfected with linearized shRNA vector together with adenoviral LacZ backbone to generate a recombinant adenovirus.

**Simulated Ischemia/Reoxygenation Protocol**

ASCs were incubated at 37 °C and 5% CO₂ for 2 h, with or without 10 µM sildenafil. This dose was selected based on its protective effect against SI/RO injury in adult cardiomyocytes (75). A subset of ASCs were treated with PKG inhibitor KT 5823 (2 µM) with or without sildenafil for 2 h. Another subset of ASCs were transduced with an adenoviral vector containing scrambled control shRNA (shRNA\textsuperscript{Con} ASC), PDE-5 shRNA (shRNA\textsuperscript{PDE-5} ASC), or PKG shRNA (shRNA\textsuperscript{PKG} ASC) in serum-free growth medium for 24 h. (Fig. 2). The cells were infected with the viruses at a concentration of 1x10\textsuperscript{3} particles/cell. ASCs were then subjected to SI for 15 h by replacing the cell medium with an “ischemia buffer” that contained 118 mM NaCl, 24 mM NaHCO\textsubscript{3}, 1.0 mM NaH\textsubscript{2}PO\textsubscript{4}, 2.5 mM CaCl\textsubscript{2}-2H\textsubscript{2}O, 1.2 mM MgCl\textsubscript{2}, 20 mM sodium lactate, 16 mM KCl, 10 mM 2-deoxyglucose (pH adjusted to 6.2) as reported previously (75). Cells were incubated in an anoxic chamber at 37°C during the entire SI period. RO was accomplished by replacing ischemic buffer with normal cell medium under normoxic conditions. Cell necrosis and apoptosis were assessed after 1 or 18 h of reoxygenation, respectively.

**Evaluation of Cell Viability and Apoptosis**

Cell viability, trypan blue exclusion assay and lactate dehydrogenase release into the medium were used to assess cell necrosis. Cell viability assessment was performed with
CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega), per the manufacturer’s instructions. In brief, approximately 5,000 ASCs/well were plated into a 96-well dish and allowed to attach overnight prior to SI/RO protocol. Following completion of SI/RO, 20μl of CellTiter 96® AQueous One Solution Reagent was added into each well and incubated for 2 h at 37°C in a 5% CO_2 before recording absorbance at 490 nm using a VersaMax microplate reader with SoftMaxPro software (Molecular Devices). CellTiter 96® AQueous One Solution uses a MTS tetrazolium compound which is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture.

ASCs were plated at a density of 2x10^5 cells/mL in 2-well chamber slides prior to SI/RO protocol. Following SI/RO, 100 μL of cellular medium was collected for LDH measurements, and enzyme activity was monitored spectrophotometrically using an LDH assay kit (Sigma). NAD+ is reduced to NADH/H+ by LDH-catalyzed conversion of lactate to pyruvate. The catalyst (diaphorase) transfers H/H+ from NADH/H+ to tetrazolium salt which is reduced to formazan. An increase in the number of dead cells leads to an increase in LDH activity in the culture medium which correlates to amount of formazan dye formed which is measured at 490 nm. Trypan blue exclusion assay was performed as follows. Following SI/RO, floating and attached cells were collected by centrifugation and cell pellets were resuspended and mixed with 20 μl of 0.4% trypan blue (Sigma). After ~ 5 min of equilibration, dead cells, stained by trypan
blue, were counted using a hemocytometer. The number of dead cells was counted from five randomly chosen fields and expressed as a percentage of the total number of cells.

Apoptosis was analyzed by the terminal dUTP nick-end labeling method (TUNEL) staining using ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore) that detects nuclear DNA fragmentation as previously reported (98). After SI and 18 h of reoxygenation, cells were fixed by 4% formaldehyde/PBS at 4 °C for 25 min and subjected to TUNEL assay according to the manufacturer’s protocol. In brief, cells were washed twice in PBS for 5 min before being permeabilized with precooled ethanol:acetic acid 2:1 for 7 min at -20°C. Cells were washed twice in PBS for 5 min before incubation 3.0% hydrogen peroxide in PBS for 5 minutes at room temperature to quench endogenous peroxidase. Cells were washed twice and incubated with terminal deoxynucleotidyl Transferase (TdT) enzyme for 1 h at 37°C in a humidified chamber. After the 1 h incubation period, cells were washed 3 times in PBS prior to incubation with anti-digoxignenin conjugate for 30 min at room temperature. Slides were developed with Nova Red peroxidase substrate (Vector Lab), counterstained with hemotoxylin and mounted using Permount solution (Fisher Scientific). Stained cells were examined under a Nikon Eclipse TE 800 microscope. TdT catalyzes a template-independent addition of nucleotide triphosphates to the 3’- OH ends of double-stranded or single-stranded DNA. The incorporated nucleotides form an oligomer composed of digoxigenin-conjugated nucleotide and unlabeled nucleotide in a random sequence. DNA fragments which have been labeled with the digoxigenin-nucleotide are then allowed to bind an anti-digoxigenin antibody that is conjugated to a peroxidase reporter molecule. The bound peroxidase antibody conjugate enzymatically generates a permanent,
intense, localized stain from chromogenic substrates, providing sensitive detection of apoptotic bodies.

**cGMP, cAMP and Protein Kinase G Activity**

cGMP activity assay was performed using cGMP Direct Immunoassay Kit (Biovision) which provided a direct competitive immunoassay for sensitive and quantitative determination of cGMP as per manufacturer’s instructions. Briefly, the cell lysate is incubated with a cGMP polyclonal antibody at room temperature for 1 h and the excess reagents are washed away. The substrate is added and after a short incubation period, the enzyme reaction was stopped and the yellow color intensity was measured using VersaMax microplate reader at 405nm. The intensity of the bound yellow color is inversely proportional to the concentration of cGMP in either standards or samples. The measured optical density is used to calculate the concentration of cGMP. Protein concentration of lysate was measured spectrophotometrically at 595 nm. The results are expressed as pmol/mg of protein.

Cellular levels of cAMP were measured using bioluminescent assay, cAMP-Glo (Promega) as per manufacturer’s instructions. In brief, approximately 5,000 ASCs/well were plated into a 96-well dish and allowed to attach overnight prior to respective treatment. Cells were lysed with incubation of cAMP-Glo Lysis buffer for 15 minutes at room temperature prior to addition of cAMP-Glo Detection Solution. After 20 min at room temperature, Kinase-Glo Reagent was added, mixed for 60 s and incubated at room temperature for 10 min. Luminescence was measured using VersaMax microplate reader. Luminescence is inversely proportional to cAMP levels. The results are expressed as pmol/mg of protein.
Cardiac protein kinase G activity was examined using a commercially available PKG activity kit (Cyclex) in whole cell lysates (n = 4/group). Activity was measured according to the manufacturer’s instructions. In brief, total soluble protein was extracted from ASCs with extraction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 50μg/ml PMSF, protease inhibitor cocktail (10μl/mL), and 0.3% β-mercaptoethanol. Cell extracts (10 μL) were plated in duplicate in a 96-well plate along with 90 μL of cGMP plus Kinase Reaction Buffer per well. The plate was covered with plate sealer, and incubated at 30°C for 30 min. Wells were washed 5 times with Wash buffer prior to addition of 100 μL of HRP conjugated Detection Antibody 10H11. The plate was recovered and incubated at room temperature for 60 min. The enzyme reaction was stopped and the spectrophotometric absorbance was measured at 450 nm. Results were normalized as per mg of protein.

**Measurement of VEGF, b-FGF, Ang-1and IGF by ELISA**

The levels of VEGF, b-FGF and IGF released from ASCs into culture medium were directly measured by ELISA kit according to manufacturer’s instructions (R & D Systems). In brief, conditioned medium was added to a microplate pre-coated with the respective growth factors for a 2 h incubation period at room temperature. Basal medium was used as a control. The plate was washed 3 times to remove any unbound substances prior to incubation with the respective enzyme-linked polyclonal antibody conjugate. Following washing to remove any unbound antibody-enzyme reagent, substrate solution is added to the wells and color develops in proportion to amount bound of respective growth factor. The absorbance was measure at 450 and 570 nm.
Measurement of Nitrate, Nitrite and NOx

ASCs were plated at a density of 2x10^5 cells/mL in 10 mm² culture dishes. ASCs were incubated at 37 °C and 5% CO₂ for 2 h, with or without 10 µM sildenafil. A subset of ASCs were transduced with an adenoviral vector containing scrambled control shRNA (shRNA\textsuperscript{Con} ASC), PDE-5 shRNA (shRNA\textsuperscript{PDE-5} ASC), in serum-free growth medium for 24 h. Conditioned medium was collected 24 h after treatment and was subsequently centrifuged using Amicon Ultra-4 centrifugal filter devices at 7500 g in 4°C to eliminate large molecules (molecular weight >30 kDa) from the medium. The levels of nitrate and nitrite in the conditioned medium were measured with a SIEVERS nitric oxide analyzer (model 280NOA). The reducing agents used were either vanadium (III) chloride (VCl\textsubscript{3}) in 1 M HCl (for nitrate) or 1% sodium iodide (NaI) in glacial acetic acid (for nitrite). Five to six mL of a reagent plus 100 µL of 1:30 diluted anti-foaming agent were loaded into the purge vessel for analysis. These reducing agents converted nitrite and nitrate respectively to gaseous NO at 90°C, which was quantified by the analyzer. The results are expressed in µM of nitrite, nitrate, and NOx (total levels of nitrate and nitrate).

Isolation of Adult Mouse Ventricular Cardiomyocytes

Adult male CD1 (Harlan Sprague Dawley) 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 (131, 132). 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°C) at a constant pressure of 55 mmHg for ~5 min with a Ca^{2+} free bicarbonate-based buffer containing: 120 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO_{4}, 1.2 mM NaH_{2}PO_{4}, 5.6 mM glucose, 20 mM NaHCO_{3}, 10 mM 2,3-butanedione monoxime, and 5 mM 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 restoration procedure (i.e. 125, 250, 500 μM Ca). The freshly isolated cardiomyocytes were then suspended in minimal essential medium (Sigma) containing 1.2 mM Ca, 12 mM NaHCO_{3}, 2.5% fetal bovine serum and 1% penicillin-streptomycin. The cells were then plated onto the 35 mm^{2} 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°C, which allowed cardiomyocytes to attach to the dish surface prior to the experimental protocol.

**In Vitro Experiments with Conditioned Medium**

Conditioned medium was generated as follows: 90% confluent ASCs were treated with or without 10 μM sildenafil for 2 h prior to being subjected to 15 h SI and 1 h RO. The medium
was then collected and used for \textit{in vitro} experiments. Adult mice cardiomyocytes were obtained from Langendorff-perfused hearts of adult CD-1 mice, as described previously. Cells were seeded in 2-well chamber slides precoated with laminin (30 µg/ml in PBS) and left to attach for 1 h. After attachment, the α-MEM medium was replaced with serum-free α-MEM (normal medium) or conditioned medium from either control or sildenafil-treated ASCs. The cardiomyocytes were then subjected to 40 min SI and 1 h RO for necrosis studies and 18 h RO for apoptosis studies.

**Cardiomyocyte Viability and Apoptosis**

Cell viability was assessed by trypan blue exclusion assay as reported previously (75, 98). Cardiomyocyte apoptosis was evaluated via TUNEL that detects nuclear DNA fragmentation via a fluorescence assay as previously reported (75, 98) using the ApoAlert™ DNA Fragmentation Assay Kit (BD Biosciences) according to manufacturer’s instructions. In brief, after SI and 18 h of reoxygenation, cardiomyocytes in two chamber slides were fixed by 4% formaldehyde/phosphate-buffered saline at 4°C for 25 min and subjected to TUNEL assay according to the manufacturer’s protocol. The slides were then counterstained with Vectashield mounting medium with 4’,6-diamidino-2-phenylindole (a DNA intercalating dye for visualizing nuclei in fixed cells, Vector Laboratories). The stained cells were examined under an Olympus IX70 fluorescence microscope. Apoptotic index (AI) was determined from counting TUNEL-positive myocyte nuclei from ten separate fields per treatment and expressed as a percentage.
Data Analysis and Statistics

Data are presented as mean ± S.E. The differences between groups were analyzed with one way analysis of variance followed by Student-Newman-Keuls post hoc test for pair-wise comparison. P<0.05 was considered to be statistically significant.
Figure 2. Experimental protocol. *In vitro* protocol, arrows indicate time points for treatment, performance of simulated ischemia/reoxygenation, and measurement of various parameters for each experimental group.
Results

Characterization of ASCs

Fluorescence activated cell sorting analysis showed that isolated ASCs expressed common surface expression markers CD29 (immune response), CD44 (cell-cell interactions, cell adhesion and migration), and CD105 (angiogenesis). The cells were devoid of markers such as CD14 (monocytes), CD45 (hematopoietic cells), and Human Leukocyte Antigen receptor DR2 (HLA-DR2) (Table 1). Immunostaining showed intense nuclear and cytosolic expression of the pluripotent stem cell transcription factors including Oct-4, Sox-2 and Nanog in the ASCs (Fig. 3A). The expression of these proteins was also confirmed by Western blot analysis. Treatment with sildenafil did not alter their expression (Fig. 3B). To demonstrate the differentiation capacity of ASCs in vitro, adipogenic and osteogenic lineage specific induction factors were used in the normal culture conditions. Adipogenic and osteogenic phenotype was determined by staining monolayers of ASCs with Oil Red-O and Alizarin Red respectively. The ASCs showed multiple intracellular lipid filled droplets in ~30% of cells confirming adipogenic differentiation (Fig. 3C) and calcium deposition with Alizarin Red confirming osteogenic differentiation (Fig. 3D).
Table 1. Surface Characterization of Isolated ASCs. Surface Marker

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Positive or Negative</th>
<th>Involvement</th>
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<tr>
<td>CD29</td>
<td>+</td>
<td>immune response</td>
</tr>
<tr>
<td>C105</td>
<td>+</td>
<td>angiogenesis</td>
</tr>
<tr>
<td>CD44</td>
<td>+</td>
<td>cell-cell interactions, cell adhesion and migration</td>
</tr>
<tr>
<td>CD14</td>
<td>-</td>
<td>monocytes</td>
</tr>
<tr>
<td>CD45</td>
<td>-</td>
<td>hematopoietic cells</td>
</tr>
<tr>
<td>Human Leukocyte Antigen</td>
<td>-</td>
<td>graft vs. host immune response</td>
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<td>receptor DR2 (HLA-DR2)</td>
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Positive results were defined as over 97% of cells expressing the surface protein of interest.
Figure 3. Expression of transcription factors and differentiation of ASCs. (A) Immunohistochemical staining of Alexa 488 labeled Oct-4 (left panel- green), Sox-2 (middle panel- green), and Nanog (right panel- green) overlayed with DAPI staining of nuclei (blue). (B) Western Blot analysis from ASC lysate show expression of stem cell transcription factors Oct-4, Sox-2 and Nanog. Treatment with sildenafil (SIL) did not alter their expression. (C) Adipogenesis and lipid vesicle formation was determined by Oil Red-O staining. (D) Mineralization was detected in osteogenic-differentiated ASCs using Alizarin red staining.
**Cytoprotection by PDE-5 Inhibition**

Immunostaining showed that ASCs express PDE-5, which was localized within the cytoplasm (Fig. 4). Moreover, adenoviral infection was confirmed by the presence of GFP expression in shRNA\textsuperscript{CON} and shRNA\textsuperscript{PDE-5}. PDE-5 expression was also confirmed by Western blot and real time-PCR. shRNA\textsuperscript{PDE-5} efficiently silenced PDE-5 in ASCs at gene and protein expression levels (Figs. 4 and 5). Adenovirally transduced or normal cultured ASCs were treated with or without 10 μM sildenafil for 2 h. The percentage of trypan blue-positive (necrotic) cells increased to 24.2±3.7 as compared to non-SI/RO controls (0.6±0.1) following SI (15 h)/RO (1 h) (n=8; p<0.01). Sildenafil treatment reduced cell death as measured by MTS-based cell incorporation (Fig. 6A) and necrosis as shown by decrease in trypan blue-positive cells to 5.7 ±1.6% (n=8; p<0.01, Fig. 5B). PDE-5 knockdown by shRNA\textsuperscript{PDE-5} conferred a similar protective effect when compared to the scrambled shRNA\textsuperscript{CON} ASCs (7.1 ±0.9 vs. 29.3 ±1.3%, p < 0.01, n = 8; Fig. 6). Also, combination of shRNA\textsuperscript{CON} with sildenafil increased cell viability and reduced necrosis (Fig. 6). Similar results were obtained when lactate dehydrogenase (LDH) release in the medium was used as a marker of necrosis in ASCs (Fig. 6C).

After 15 h of SI and 18 h of RO, apoptotic nuclei (TUNEL-positive cells) increased from 2.0±0.5 % (in non-ischemic control group) to 18.3±2.5 % of total cells (p < 0.01, n = 8;). PDE-5 inhibition resulted in reduction of TUNEL-positive cells to 5.7± 2.1% in sildenafil-treated ASCs and 6.4± 0.9% in shRNA\textsuperscript{PDE-5} ASCs (p < 0.01 vs. SI-RO, n = 8; Fig. 7). These results suggest that PDE-5 inhibition in ASCs exerts a cytoprotective against ischemic injury.
Figure 4. PDE-5 immunofluorescence in ASCs. Immunohistochemical staining of PDE-5 (red), GFP (green), DAPI staining of nuclei (blue) and overlay in ASCs with shRNA\textsuperscript{PDE-5} as compared to shRNA\textsuperscript{CON} ASCs and control ASCs.
Figure 5. Expression of PDE-5 in ASCs (A) Western blot analysis showing knock-down of PDE-5 in ASCs with shRNA^{PDE-5} as compared to shRNA^{CON} ASCs and control ASCs (representative lanes from contiguous blot; n=3). (B) Real-time PCR showing reduced PDE-5 expression; (n=3).
Figure 6. Effect of PDE-5 inhibition on protection of ASCs from necrosis. Quantitative data showing the effect of sildenafil (SIL) or shRNA$_{\text{PDE-5}}$ on necrosis following SI-RO as determined by MTS cell viability assay (A), trypan blue staining (B), and LDH release (C); (n=8).
Figure 7. Effect of PDE-5 inhibition on protection of ASCs from apoptosis. Quantitative data showing the effect of sildenafil (SIL) or shRNA\textsuperscript{PDE-5} on apoptosis following SI-RO as determined by TUNEL assay (A) and representative images (B); n=8).
Effect of PDE-5 Inhibition on cGMP and PKG Activity

PDE-5 inhibition by sildenafil or shRNA$^{\text{PDE-5}}$ resulted in nearly identical increase in cGMP levels (0.9±0.01 and 0.9±0.02 pmol/mg of protein, respectively) as compared to non-treated ASCs (0.7±0.03) and shRNA$^{\text{CON}}$ ASCs (0.7±0.03) (p<0.05, n=4). shRNA$^{\text{CON}}$ had no effect on cGMP formation with sildenafil treatment (Fig. 8A). Both sildenafil and shRNA$^{\text{PDE-5}}$ had no effect on cAMP levels (Fig 8B). Also, sildenafil and shRNA$^{\text{PDE-5}}$ increased PKG enzymatic activity (A450/mg protein) as compared to control ASCs (Fig. 8D).

To determine the cause and effect relationship of PKG in sildenafil-induced survival of ASCs following SI/RO, we used shRNA knockdown of PKG and pharmacological inhibition approach as reported previously (99). ASCs infected with shRNA$^{\text{PKG}}$ caused at least 60% knock-down of PKG (Figs. 8C). Moreover, sildenafil induced PKG activity was inhibited in shRNA$^{\text{PKG}}$ ASCs or by co-treatment with PKG inhibitor, KT 5823 (Fig. 8D). The protective effect of sildenafil against necrosis and apoptosis (Figs. 6, 7) were attenuated by KT 5823 and in shRNA$^{\text{PKG}}$ ASCs (Figs. 9, 10) suggesting that inhibition of PDE-5 induced protection involves a PKG dependent pathway.
Figure 8. PDE-5 inhibition increases cGMP and PKG activity. (A) cGMP levels in ASCs following preconditioning with sildenafil and shRNA\textsuperscript{PDE-5} as compared to shRNA\textsuperscript{CON} and nontreated ASCs. (B) cAMP levels in the same groups as in A. (C) Western blot analysis showing knockdown of PKG expression as compared to controls (D) PKG activity in ASCs following treatment with sildenafil or shRNA\textsuperscript{PDE-5}. Treatment with 2 µM KT 5823 (KT) or shRNA\textsuperscript{PKG} inhibited PKG activity; (n=4).
Figure 9. Sildenafil protects ASCs against necrosis through PKG dependent mechanism. Quantitative data showing cell viability as determined by MTS assay (A), cell necrosis assessed by trypan blue exclusion assay (B), and LDH release into the medium (C); (n=8).
Figure 10. Sildenafil protects ASCs against apoptosis through PKG. Quantitative data showing the effect of PKG inhibitor KT 5823 (KT) and shRNA\textsubscript{PKG} on apoptosis following SI-RO as determined by TUNEL assay (A) and representative images (B). The PKG inhibitor KT 5823 (KT) and shRNA\textsubscript{PKG} abolished the protective effect of sildenafil against necrosis and apoptosis; (n=8).
PDE-5 Inhibition Enhances Release of Growth Factors

To identify potential paracrine mechanisms responsible for the therapeutic effect of preconditioning, we examined the effect of PDE-5 inhibition in ASCs on release of growth factors, VEGF, b-FGF, and IGF-1 \textit{in vitro}. No differences in their secretion was observed between sildenafil-treated, shRNA$^{\text{PDE-5}}$ and non-treated ASCs under normal conditions. Following SI/RO, both sildenafil and knockdown with shRNA$^{\text{PDE-5}}$ increased the release of basic-fibroblast growth factor (b-FGF) (1.7 fold), IGF-1 (1.5 fold) and VEGF (1.4 fold) as compared to SI/RO control. Inhibition of PKG blocked the enhanced secretion of the growth factors (Fig. 11). These data suggests that the high-level secretion of growth factors from preconditioned ASCs may provide cardioprotective and proangiogenic effects as a result of PKG activation.
Figure 11. PDE-5 inhibition increases the release of growth factors following ischemia/reoxygenation in ASCs. PDE-5 inhibition by sildenafil or its knockdown with silencing vector shRNA_{PDE-5} augmented the release of (A) b-FGF, (B) IGF, and (C) VEGF after SI/RO which is blocked by PKG inhibitor KT 5823 (KT) and shRNA_{PKG}. shRNA_{CON} had no effect; (n=4).
Effect of Sildenafil Preconditioning on Nitrate, Nitrite and NOx

The concentrations of nitrate, nitrite and NOx (nitrate + nitrite) secreted into culture medium was examined following PDE-5 inhibition in ASCs. Sildenafil treatment of ASCs resulted in significant increases of nitrate (26±1.7 μM) and NOx (30.5±0.8 μM) as compared to non-treated controls (17.9±1.7 μM and 22.3±1.7 μM, respectively, p<0.05, n=3). Interestingly, PDE-5 inhibition through a silencing vector exhibited a trend towards enhanced secretion of nitrate and NOx as compared to non-treated ASCs; however, did not confer a similar increase as compared to ASCs infected with scrambled control vector (Fig. 12). Furthermore, the levels of nitrite secreted into the medium were similar in each treatment group.
Figure 12. Increased NOx levels following sildenafil treatment. Quantitative data showing the effect of sildenafil (SIL) or shRNA^{PDE-5} on levels of nitrate (A); nitrite (B); and NOx (C) in conditioned medium; (n=3).
Cytoprotection of Adult Mouse Cardiomyocytes with Conditioned Medium:

We further evaluated the possible paracrine effects of conditioned medium from cultured ASCs on isolated primary adult cardiomyocytes. Our method for isolation and cell preparation yielded at least 85% of the cardiomyocytes with rod-shaped morphology (Fig. 13 B). Initially, the mouse cardiomyocyte standard growth medium was replaced with normal α-MEM or conditioned medium from control or sildenafil-treated ASCs; cardiomyocytes were subsequently subjected to SI-RO (Fig. 13 A). Necrosis was measured by trypan blue exclusion and LDH release. The percentage of trypan blue-positive (necrotic) cardiomyocytes increased to 41.3±5.8% as compared to non-SI/RO controls (2.0±0.8%) following SI (40 min)/RO (1 h) (p<0.01, n=4). Treatment with conditioned medium (CM) reduced necrosis as shown by decrease in trypan blue-positive cells. Although the cytoprotective effect was observed in the presence of both control and sildenafil-treated ASC-CM, the greater degree of protection was conferred by the SIL ASC-CM (24.5±2.6% vs 32.3 ±3.9%, p<0.01, n=4, Fig. 13 C). Similar results were obtained by measurement of LDH release in the medium (Fig. 13 D). Furthermore, the relative number of apoptotic cardiomyocytes following 18 h of RO was measured by TUNEL analysis. In the presence of SIL ASC-CM, the number of apoptotic nuclei was reduced to 11.5±2.9% as compared with that of normal medium (26.3±4.2%) or the control ASC-CM (18.5±2.1%), respectively (Fig. 14). Thus, these results indicate that a paracrine cytoprotective mechanism is involved with growth factors secreted by the ASCs under ischemic conditions.
A. Group 1- Control  
Group 2- SI-RO + control medium  
Group 3- SI-RO + ASC conditioned medium (ASC-CM)  
Group 4- SI-RO + Sil ASC conditioned medium (SIL ASC-CM)  

1 hour treatment of isolated cardiomyocytes with medium prior to SI/RO

40 min Simulated Ischemia  
Reoxygenation

1 hour  
• Trypan Blue Exclusion  
• LDH release

18 hours  
• TUNEL

B. Figure 13. Conditioned media attenuates necrosis of cardiomyocytes following ischemia/reoxygenation. (A) In vitro protocol, arrows indicate time points for treatment, performance of simulated ischemia/reoxygenation, and measurement of various parameters for each experimental group. (B) Representative image of isolated mouse cardiomyocytes. Quantitative data showing cell necrosis assessed by trypan blue exclusion assay (C), and LDH release into the medium (D); (n=4).
Figure 14. Conditioned media attenuates apoptosis of cardiomyocytes following ischemia/reoxygenation. Quantitative data showing the effect of conditioned medium from sildenafil-treated ASCs (SIL ASC-CM) or control ASCs (ASC-CM) on apoptosis following SI-RO as determined by TUNEL assay (A) and representative images (B); (n=4).
Discussion

Cardiac repair via cellular transplantation has generated considerable enthusiasm in recent years although the optimal cells for cardiac repair remain to be identified. We chose adipose-derived stromal/stem cells which have the ability to differentiate into multiple mesenchymal cell types including endothelial cells (45, 46) and cardiomyocytes (15, 52). Human ASCs have been shown to preserve heart function following myocardial infarction (133). In the present study, we investigated the feasibility of PDE-5 inhibition as a strategy to precondition human ASCs. The rationale for this approach was the established powerful preconditioning-like effect of PDE-5 inhibitors in cardiomyocytes (75, 98) and against ischemia/reperfusion injury in heart (92-94) established by us. Our results show preconditioning of ASCs by PDE-5 inhibition significantly improved their ability to survive SI/RO injury in vitro. Moreover, we observed significant release of pro-angiogenic/pro-survival growth factors including VEGF, b-FGF, and IGF-1. More importantly, we provide the first evidence for robust expression of PDE-5 in the isolated ASCs. PDE-5 knockdown with a silencing vector significantly reproduced the effect of sildenafil in survival against cell death as well as release of growth factors. These data not only rule out the potential off target effects of sildenafil in ASCs but also provide us a genetic approach to precondition the stem cells possibly for improving survival after transplantation. To our knowledge, this is the first study showing PDE-5 as a target gene/enzyme to improve survival of ASCs under ischemic conditions in vitro. These are clinically significant observations because improving stem cell survival by exploiting novel therapeutic targets with clinically approved drugs would directly impact the prognostic outcome of stem cell therapy in the heart.
We have shown that sildenafil reduced myocardial infarction in the intact heart and apoptosis and necrosis in cardiomyocytes subjected to simulated ischemia and reoxygenation through nitric oxide (NO)-dependent pathway (75). Sildenafil induced a delayed cardioprotective effect in the mouse heart that involved the upregulation of iNOS and eNOS (91). ASCs have been shown to endogenously generate NO which can be measured in biological systems as metabolites of NO such as nitrite and nitrate (134). Our results indicate a significant increase in concentrations of nitrate and NOx (nitrate + nitrite) in culture medium following sildenafil treatment of ASCs as compared to non-treated cells. Interestingly, shRNA\textsuperscript{PDE-5} ASCs exhibited a trend towards increased NO generation as compared to control ASCs; however, there was not a significant difference in NO metabolites as compared the shRNA\textsuperscript{CON} ASCs. The downstream targets and cellular actions of NO are known to be dependent on its local concentrations with normal in vivo concentrations of NO ranging from low nanomolar to low micromolar level. Therefore, even modest increases in NO concentration can result in activation of the major NO receptor, soluble guanylyl cyclase which in turn elevates intracellular cGMP (101). Enhanced accumulation of cGMP leads to activation of PKG which has been shown to be involved in cardioprotective effects against I/R injury (102-104). In the present study, there is no clear evidence that PKG activation seen in preconditioning of ASCs is a result of increased NO production, although recently, we demonstrated that direct adenoviral overexpression of PKG I\textalpha in cardiomyocytes induced both iNOS and eNOS and protected these cells against SI/RO injury. Similarly in isolated cardiomyocytes (75), sildenafil caused a significant increase in mRNA and protein expression of iNOS and eNOS. Also, sildenafil-induced protection against necrosis and apoptosis was abolished in cardiomyocytes derived from iNOS but not from eNOS gene
knockout mice (91). ASCs have been shown to express all three nitric oxide synthases, iNOS (135), eNOS (45, 136), and nNOS (137), and endogenous generation of NO has been attenuated with the iNOS/eNOS inhibitor, L-NAME (134). Therefore, it is plausible that the increase in NO concentrations following sildenafil treatment might be a result of a similar mechanism involving iNOS/eNOS up-regulation. However, further studies are needed to delineate the exact source of NO in our model of ASC preconditioning with sildenafil.

Inhibition of PDE-5 by siRNA has been documented to increase levels of cGMP and increase the angiogenic phenotype of endothelial cells (98, 130). PDE-5 inhibition and activation of cGMP has also been shown to increase mobilization and exert pro-survival effects on stem cells (138, 139). In ours and other previous studies, PDE-5 inhibition lead to accumulation of cGMP to levels that lead to activation of PKG. Phosphorylation of downstream proteins by PKG regulates such important physiological functions as relaxation of vascular smooth muscle, inhibition of platelet aggregation and apoptosis, and induction of VEGF (15, 97). We have demonstrated previously that sildenafil induced activation of PKG and adenoviral overexpression of PKG in isolated cardiomyocytes resulted in attenuation from necrosis and apoptosis following ischemia/reoxygenation (75, 114). The present results demonstrate that preconditioning of ASCs with PDE-5 inhibition exerted a similar cytoprotective effect through a PKG-dependent pathway as PKG inhibitor, KT 5823, and short-hairpin RNA knock-down of PKG abolished protection.

There are at least two mechanisms contributing to the resistance of ASCs in which PDE-5 is inhibited against hypoxic/ischemic stress. We have previously shown that sildenafil induces a cytoprotective effect in cardiomyocytes through NO and cGMP dependent activation of PKG which results in opening of mitochondrial KATP channels (75, 98, 140). While the
comprehensive cytoprotective signaling pathways following PDE-5 inhibition in ASCs remains to be investigated, it is clear that cGMP (but not cAMP) was elevated and PKG was involved in protecting ASCs against ischemic injury (Figs. 8, 9, 10). PKG is a serine/threonine protein kinase that has two isozymes (type I and type II; i.e. PKGI and PKGII). PKGIα is mainly found in lung, heart, platelets, and cerebellum, whereas PKGIβ is highly expressed in smooth muscles of uterus, vessels, intestine, and trachea (141). As discussed elsewhere, sildenafil activated PKG-dependent signaling cascade that involved phosphorylation of ERK and inhibition of GSK-3β thus leading to cytoprotection (98). Moreover, gene transfer of PKGIα in cardiomyocytes in the absence of sildenafil or other pathophysiological stimuli (such as ischemic preconditioning) resulted in a cytoprotective phenotype that was associated with the phosphorylation of Akt, ERK, and JNK and increased Bcl-2 expression (114). It is quite likely that a similar cascade of signaling events leads to survival of ASCs after SI.

Another cytoprotective mechanism of PDE-5 inhibition in ASCs may involve the paracrine effects by secretion of growth factors with angiogenic potential. The present study clearly demonstrated the increased secretion of VEGF, b-FGF, and IGF-1 from sildenafil- and shRNA\textsubscript{PDE-5}-treated ASCs in response to the ischemic conditions. Moreover, the release of growth factors was blunted under conditions where PKG signaling was disrupted (Fig. 6) suggesting a critical role of cGMP-PKG pathway in their secretion. These results are supported by other studies on downregulation of VEGF expression with the inhibition of the downstream kinase of PKG, GSK3β (142), which are also activated by sildenafil treatment in cardiomyocytes and exert protective effect against SI/RO injury (98). ASCs have been shown to secrete VEGF and HGF which possess both angiogenic and anti-apoptotic effects on both myocardial and
endothelial cells (47). Furthermore, these paracrine factors from stem cells have been shown to protect co-cultured adult cardiomyocytes against hypoxic/ischemic stress (144-146). Accordingly, we observed that adult mouse cardiomyocytes cultured with conditioned medium from sildenafil treated ASCs survived better than those cultured with conditioned medium from non-treated cells. Therefore, transplantation of ASCs preconditioned through PDE-5 inhibition could provide adequate magnitude and duration of VEGF, b-FGF, and IGF-1 release in the ischemic myocardium, which would provide cardioprotective effects leading to the salvaging of ischemic myocardium and decrease the infarcted area.

In summary, this study demonstrates that preconditioning of ASCs through PDE-5 inhibition, improved survival under conditions of ischemia/reoxygenation via PKG activation and enhanced paracrine action. We propose that in vitro preconditioning of ASCs by inhibition of PDE-5 with small molecule drugs or gene silencing vectors can be a powerful new approach to improve stem cell therapy following myocardial infarction in patients. Particularly the easy availability of ASCs from humans combined with the preconditioning by inhibition of PDE-5 may hold great promise for initiation of clinical trials in heart failure patients.

**Funding Sources:** This study was supported by grants from National Institutes of Health (HL51045, HL79424 and HL93685) to Rakesh C. Kukreja, National Scientist Development Grant from the American Heart Association (10SDG3770011) to Fadi N. Salloum, and Predoctoral Fellowship from the American Heart Association (09PRE2250905) to Nicholas N. Hoke.
ABSTRACT

Cell-based therapies for the prevention and treatment of cardiac dysfunction offer the potential to significantly alter cardiac function and improve the outcome of patients with cardiovascular disease. To date several clinical studies have suggested the potential efficacy of stem cell therapy; however, the benefits have been limited and inconsistent. We have demonstrated that preconditioning of adipose-derived stem cells (ASCs) through PDE-5 inhibition improves their viability following simulated ischemia and re-oxygenation. We therefore tested the hypothesis that ex vivo preconditioning of ASCs with sildenafil (Viagra) or selective knockdown with a silencing vector would enhance cardiac function following myocardial implantation in vivo. ASCs were preconditioned by treatment with sildenafil or PDE-5 silencing vector shRNA (shRNA\textsuperscript{PDE-5}). Adult male CD-1 mice underwent myocardial infarction (MI) by occlusion of left descending coronary artery. Animals received PBS and preconditioned ASCs (4x10\textsuperscript{5}) by direct intramyocardial injection into left ventricular wall. Cardiac function and structure were evaluated by serial echocardiography and histology. Preconditioned ASCs-treated hearts showed consistently superior cardiac function by all measures as compared with PBS and non-
preconditioned ASCs 4 weeks after treatment. Post-mortem histological analysis demonstrated that preconditioned ASCs-treated mice had significantly reduced fibrosis, increased vascular density and decreased resident myocyte apoptosis as compared to mice that received non-preconditioned ASCs or PBS. Plasma levels of VEGF, b-FGF, and Ang-1 were also significantly elevated 4 weeks after cell therapy with preconditioned ASCs. We conclude that ex vivo inhibition of PDE-5 prior to transplantation can be a potent new approach to improve stem cell therapy following myocardial infarction.
Introduction

Over the past several years, experimental findings suggest there is a therapeutic potential for cellular replacement therapy as treatment of acute myocardial infarction (MI) and other progressive chronic cardiac diseases such as left ventricular (LV) remodeling and heart failure. Since cardiovascular disease remains a worldwide problem, the development of novel effective cell-based therapies is crucial to improve patient outcome post MI. Current therapies strive to limit both the loss of cardiomyocytes and adverse remodeling in order to preserve cardiac systolic function; however, treatment of MI still leaves a significant number of patients with impaired cardiac function that leads to more severe LV dysfunction and adverse remodeling. Remodeling of the ventricle is a result of increased cardiomyocyte apoptosis in the ischemic zones after infarction. While apoptosis influences remodeling, the other form of cellular death that occurs in the heart, necrosis, provokes inflammatory reactions, neoangiogenesis, fibroblast activation, and scar formation. Therefore, limiting myocardial apoptosis and necrosis is of critical importance.

Phase II and III clinical studies indicate that stem cell transplantation is feasible and may have beneficial effects on ventricular remodeling after myocardial infarction. However, to date, most of the cellular based therapies have shown limited efficacy because the majority of transplanted cells are readily lost after transplantation due to poor blood supply, ischemia/reperfusion injury, and inflammatory factors. Disconcerting reports have shown that up to 99% of transplanted cells are lost within the first 24 hours (73). Therefore, enhancing cell viability and reduction of apoptosis of ASCs in an ischemic microenvironment of the infarcted heart is critical for improving the efficiency of cell therapy. In the previous chapter, we have
demonstrated that in vitro PDE-5 inhibition of ASCs by sildenafil or genetic knock-down with a silencing vector improved cell viability, attenuated necrosis and apoptosis following simulated ischemia/reoxygenation (SI/RO) through a PKG-dependent mechanism. Moreover, we demonstrated the increased secretion of pro-angiogenic/pro-survival growth factors: VEGF; bFGF; and IGF-1 from sildenafil and shRNA\textsuperscript{PDE-5} -treated ASCs in response to the ischemic conditions. Transplantation of ASCs along with their secretion of VEGF, bFGF, and IGF-1, and has been shown to upregulate the anti-apoptotic protein, Bcl-2, which results in the decrease in cardiomyocyte apoptosis (58). Similarly, we showed that treatment with conditioned medium from sildenafil-treated ASCs resulted in a cytoprotective effect on cultured adult mouse myocytes following SI/RO through attenuation of necrosis and apoptosis.

In this study, we tested the hypothesis whether PDE-5 inhibition could improve the survival and engraftment of ASCs which may lead to enhanced cardiac function following myocardial infarction in mice. Specifically we addressed the question: Whether in vivo transplantation of ASCs after ex vivo PDE-5 inhibition improve LV function following myocardial infarction. Our results show that ex vivo PDE-5 inhibition of ASCs prior to myocardial transplantation enhances their therapeutic potential as shown by reduced fibrosis, cardiomyocyte apoptosis, improved vascular density and cardiac function in mice following myocardial infarction.
Materials and Methods

Animals

Adult male outbred CD-1 mice (~30g) were supplied by Harlan Sprague Dawley. Upon their arrival, the animals were allowed to readjust to the housing environment for at least 3 days before any experiment. Standard food and water were freely accessible for the animals used in these studies. 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) Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 80-23; Office of Science and Health Reports, Bethesda, MD 20205].

Myocardial Infarction (MI) Protocol

Adult CD-1 mice underwent permanent occlusion of the left descending coronary artery as previously described (97). In brief, the animals were anesthetized with pentobarbital (70 mg/kg ip), intubated orotracheally and ventilated on a positive-pressure ventilator. The tidal volume was set at 0.2 ml, and the respiratory rate was adjusted to 133 cycles/min. All surgical procedures were carried out under sterile conditions. A left thoracotomy was performed at the fourth intercostal space, and the heart was exposed by stripping the pericardium. The left descending coronary artery (LAD) was then identified and permanently occluded by a 7.0 silk ligature that was placed around it. After coronary artery occlusion, the air was expelled from the chest. The animals were extubated and then received intramuscular doses of analgesia (buprenex; 0.02mg/kg) and antibiotic (Gentamicin; 0.7 mg/kg; for 3 days).
Transplantation of ASCs

Immediately after ligation, $4 \times 10^5$ ASCs (total of $30 \mu$L) were injected at 3 injection sites into anterior and lateral wall of the LV bordering the infarction. The control group was injected with identical volume of PBS at similar sites (Fig. 1). To determine the fate of ASCs post transplantation, the cells were labeled with PKH26 dye using a PKH-26 Cell Linker Kit (Sigma) according to the supplier’s instructions. The chests were sutured, and animals were allowed to recover. The hearts were harvested for histological studies 4 weeks post cellular transplantation.
Group 1- Sham
Group 2- MI + PBS
Group 3- MI+ ASC
Group 4- MI + Sil ASC
Group 5- MI + shRNA\textsuperscript{CON} ASC
Group 6- MI + shRNA\textsuperscript{DG-5} ASC

Figure 15. Experimental protocol. In vivo protocol, arrows indicate time points for treatment, performance of surgical procedures, and measurement of various parameters for each experimental group. Arrowheads indicate sites of injection of adipose-derived stem cells in the border zone of the infarcted heart.
Echocardiography

Doppler Echocardiography was performed using the Vevo770TM imaging system (VisualSonics Inc., Toronto, Canada) prior to surgery (baseline), and 4 weeks after surgery using techniques similar to those previously reported (97). Mice were anesthetized with pentobarbital sodium (30 mg/kg ip). The mice were placed in the supine position. The chest was carefully shaved, and ultrasound gel was used on the thorax to optimize visibility during the exam. A 30-MHz probe was used to obtain two-dimensional (B-Mode), and cross-sectional (M-mode) imaging from parasternal short-axis view at the level of the papillary muscles. The M-mode cursor was positioned perpendicular to the anterior and posterior wall to measure left ventricular (LV) end-diastolic and end-systolic diameters (LVEDD and LVESD, respectively). M-mode images were then obtained at the level of the papillary muscles below the mitral valve tip. LV fractional shortening (FS) was calculated as follows: FS=(LVEDD–LVESD)/LVEDDx100. Ejection fraction was calculated with the Teichholz formula.

Histology

Following the 4 week post-MI echo, the mice were sacrificed and subsequently, the hearts were excised, trimmed free of the atria, vena cava, and pulmonary vessels, and prepared for histology. In brief, the aorta was attached to a Langendorff apparatus and the heart was perfused in retrograde fashion with saline to remove the residual blood. Tranverse sections of the median third of the LV (n = 4-6/group) were flash frozen in liquid nitrogen and sent to VCU Tissue and Data Acquisition and Analysis Core (TDAAC) Facility for tissue processing and
cryosectioning. Apoptosis was examined using TUNEL assay according to manufacturer’s instructions which were described in Chapter 2. The apoptotic rate was expressed as the number of apoptotic cells of all cardiomyocytes per field. The apoptotic rate in the peri-infarct regions was calculated using 10 random fields, which virtually cover the entire peri-infarct area.

Myocardial fibrosis was examined to address prevalence of scar formation within the LV. Heart sections (5 μm) were stained with Masson’s trichrome (Sigma). Fibrotic area was computed using computer morphometry (Bioquant) and expressed as a percentage of left ventricle.

**Determination of Vascular Density**

The vascular density in the peri-infarct region of myocardial tissue was determined as previously described (147). In brief, tissue sections were stained using CD-31 antibody (Millipore) for evaluation of vascular density. Blood vessels were quantified using histomorphometry. Vessels were distinguished from larger arterioles and venules by deriving lumen diameters from area assessments of each vessel. Only vessels with diameters <10 μm and cut in an orthogonal fashion were considered and included in the quantification. For quantification of positively stained vessels, five sections within the peri-infarcted area of each animal were analyzed. Blood vessels were detected at low magnification, x 200.
Data Analysis and Statistics

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

PDE-5 Inhibition Enhances ASC Survival in Infarcted Hearts

To determine the engraftment of ASCs, we examined myocardial tissue sections for the presence of PKH26 positive cells. Donor PKH26 positive cells were identified only in mice that received ASCs after treatment with sildenafil or shRNA\textsuperscript{PDE-5}. PKH26-positive cells were present primarily within the epicardial layer of the infarct border zone region (Fig. 16). There was no evidence of engrafted ASCs in mice receiving control non-preconditioned ASCs. To determine endothelial cell differentiation of ASCs, we immunostained heart sections with endothelial cell specific marker, CD31, and assessed for co-localization of PKH26 positive cells. Animals receiving ASCs treated with sildenafil or shRNA\textsuperscript{PDE-5} showed CD31 positive cells.
Figure 16. Tracking of PKH26 labeled ASCs following myocardial infarction. ASCs were preconditioned with sildenafil (Sil) or shRNA^{PDE-5} and injected in the LV wall following myocardial infarction. (A) PKH26 labeled ASCs Labeled (red) transplanted Sil or shRNA^{PDE-5} ASCs. DAPI (blue) was used to visualize nuclei. (B) Immunostaining of CD31 (green), PKH26 (red) and DAPI (blue).
Preconditioned ASCs Improve Cardiac Function, Reduce Fibrosis and Apoptosis

Figure 17A shows representative M-mode images 4 weeks after MI. Significant functional loss continued over the following 28 days in saline-treated hearts. Echocardiography recordings show that values for all measures of LV function decreased significantly in hearts treated with PBS post MI as compared with sham. Conversely, hearts injected with ASCs demonstrated a trend toward an increase in function. However, treatment with preconditioned ASCs demonstrated significant improvement in function as compared with non-preconditioned cells or PBS. Specifically, LVEDD, LVESD, EF, and FS were improved significantly in mice receiving sildenafil-treated ASCs as compared with mice receiving non-treated ASCs at 4 weeks after MI (Fig. 17). Administration of ASCs preconditioned with shRNA$^{PDE-5}$ also caused similar enhanced preservation of cardiac function and attenuation of cardiac remodeling as compared to ASCs treated with control vector. There were no significant differences in the echo parameters in mice receiving shRNA$^{CON}$ ASCs and non-treated ASCs (Fig. 17).

The percentage of fibrosis in the LV wall was 55 to 60% lower in hearts receiving ASCs preconditioned with PDE-5 inhibition as compared to non-treated ASCs (7.1±0.7% with sildenafil-treated ASCs; 6.8±1.7% with shRNA$^{PDE-5}$ ASCs vs 14.9±3.8% with non-treated ASCs, p < .05, n=5). All cellular treatment groups showed significant reduction in fibrosis compared with PBS controls (31.3±3.8%, Fig. 18). Also, the reduction in fibrosis seen in sildenafil-treated and shRNA$^{PDE-5}$ ASCs correlated with a reduction of cardiomyocyte apoptosis. PBS-treated mice after 4 weeks of permanent occlusion had a resident myocyte apoptotic rate of 3.3±0.32%, which was reduced to 1.9±0.23% in non-treated ASC group. Sildenafil-treated or
shRNA\textsuperscript{PDE-5} ASCs injected mice further reduced apoptosis (0.9±.14% with sildenafil and 0.8±.12% with shRNA\textsuperscript{PDE-5} ASCs, p<0.05 vs. PBS control, n=5, Fig. 19).
Figure 17. Transplantation of preconditioned ASCs improves cardiac function and remodeling following myocardial infarction. (A) Representative M-mode images showing preservation of LV contractility of hearts treated with preconditioned ASCs as compared with non-treated ASCs control following myocardial infarction (MI). Bar diagram showing quantitative data of hearts treated with preconditioned ASCs as compared with non-treated ASCs control following myocardial infarction. (B) LV end-diastolic diameter; (C) end-systolic diameter; (D) fractional shortening; (E) ejection fraction; (n≥5).
Figure 18. Preconditioned ASCs reduces myocardial fibrosis following myocardial infarction. (A) Representative Masson’s trichrome staining of tissue sections and accompanying (B) bar diagram showing quantification of the amount of fibrosis (n=4).
Figure 19. Preconditioned ASCs reduces myocardial apoptosis following myocardial infarction. (A) Representative TUNEL staining of sections and (B) bar diagram showing quantification of TUNEL positive cells; (n=4).
Preconditioned ASCs Increase Vascular Density and Secretion of Growth Factors

Rapid restoration of blood supply to the ischemic region is critical for stabilizing the border region of the infarct and supporting viable and regenerating myocardium. We used CD-31 positive staining to determine the vascular density in the border zone of MI. Transplantation of ASCs significantly increased vascular density as compared with that of PBS-treated hearts (Fig. 20). However, the vascular density in sildenafil and shRNA\textsuperscript{PDE-5} ASCs treated mice was significantly enhanced as compared to non-treated ASCs mice (8.3±1.3 in sildenafil-treated ASCs; 7.8±0.5 shRNA\textsuperscript{PDE-5} ASCs vs 4.5±0.6 vessels/HPF, p<0.01, n=4) (Fig. 20). Moreover, the plasma levels of b-FGF, Ang-1, and VEGF were significantly increased in mice receiving preconditioned ASCs (with sildenafil-treated or shRNA\textsuperscript{PDE-5} ASCs) as compared to non-treated ASCs (p<0.001, n=5, Fig. 21).
Figure 20. Preconditioned ASCs increases vascular density following myocardial infarction. (A) Representative images and (B) bar graph depicting immunostaining of sections with CD31 to assess vascular density; (n=4).
Figure 21. Transplantation of preconditioned ASCs enhances release of growth factors following myocardial infarction. Bar diagram showing blood levels of (A) b-FGF, (B) VEGF and (C) Ang-1 from the sham and infarcted hearts treated with PBS (MI); non-preconditioned ASCs (MI+ASC); preconditioned with sildenafil (MI+Sil ASCs) or PDE-5 silencing vector, shRNA^{PDE-5} (MI+ shRNA^{PDE-5}) 4 weeks after MI. Note that preconditioned ASCs enhanced release of b-FGF, VEGF and Ang-1 as compared to control hearts; (n=5).
Discussion

Current treatment of MI still leaves a significant number of patients with impaired cardiac function that leads to more severe LV dysfunction as the ventricle remodels. Thus, cell therapy has generated much excitement as a novel therapy that might provide additive benefits over conventional treatment to restore or prevent further LV dysfunction after MI. Although there have been several reports that ASCs improve cardiac function post-MI effects have been limited (69-72). This study investigated the feasibility of ex vivo preconditioning by PDE-5 inhibition as a strategy to enhance the efficacy of stem cell therapy post-MI. As described in Chapter 2, PDE-5 inhibition in ASCs confers a powerful preconditioning-like effect against SI/RO limiting apoptosis and necrosis thus increasing survivability. The present study shows significant improvement in cardiac function 4 weeks after transplantation of preconditioned ASCs as demonstrated by significant preservation of FS and EF as compared to other treatment groups. Moreover, the superior functional improvement compared with non-preconditioned ASC injected group was associated with enhanced vascular density (Fig. 20), decrease in fibrosis and resident cardiomyocyte apoptosis (Fig. 18, 19). The observed benefits correlated with increased plasma levels of pro-angiogenic growth factors including VEGF, b-FGF, and Ang-1.

Various studies have reported the beneficial effects of stem cell therapy without long-term engraftment, and a link between donor cell-derived factors and LV recovery following cellular transplantation (67, 148-150). In the present study, we also observed a very limited number of PKH26 positive ASCs cells that were identified only in hearts which received preconditioned ASCs. PKH26-positive cells were present primarily in the epicardium of the border zone regions after 4 weeks (Fig. 16). The observation that only a limited number of
preconditioned cells were present 4 weeks post-MI is consistent with other studies that suggest that improving the effectiveness of graft patency with stem cells is dependent on increased concentrations of NO (151). We observed an increase in NO generation following sildenafil treatment (Chapter 2). Furthermore, other studies have showed a rapid loss of transplanted cells after 24 h and 7 d following direct intramyocardial injection post-MI. Moreover similar to our study, it was reported that the numbers of cells present in the post-MI heart were almost negligible by 2 weeks. However, cellular transplantation still attenuated LV dysfunction and remodeling, reduced myocyte apoptosis and augmented myocardial neovascularization, despite poor engraftment of transplanted cells (152). Another study by MacLellan et al (51) demonstrated the ability of ASCs, delivered acutely following LAD ischemia/reperfusion, to improve cardiac function independent of engraftment. This is in agreement with Limbourg et al. who also showed that hematopoietic stem cells can improve cardiac function post-MI, even in the absence of sustained engraftment (153). Interestingly, similar reductions in fibrotic tissue have been observed when either the supernatant from the modified cells or specific secreted proteins used to modify the mesenchymal stem cell were injected into the infarct zone (66). These observations support the paracrine hypothesis of myocardial repair, and suggest not only the potential for pharmacological or genetic enhancement of stem cell therapies, but also suggest that high degree of benefit observed with cell therapy is independent of cellular engraftment. As cellular differentiation does not appear to play a major role in the therapeutic effect of ASCs in this model system, it is logical to hypothesize that the paracrine release of cytokines and growth factors by the transplanted ASCs in the first few days or weeks after injection is responsible for the observed effects. Consistent with our results, it is possible that a small number of specifically
conditioned or genetically altered cells may be able to exploit paracrine pathways to maximize the biological and clinical effects after cell delivery (9, 58, 148, 154-159). Paracrine factors released by modified or preconditioned ASCs potentially mediate multiple mechanisms such as myocardial cell survival, remodeling, contractility, increased blood flow to ischemic tissue, regulation of inflammatory response, and recruiting endogenous stem cells to regenerate injured tissue. The lack of significant engraftment despite tremendous reduction in cardiac fibrosis seen in the preconditioned ASC treatments suggests that a paracrine mechanism from enhanced secretion of growth factors may play an important beneficial role in repairing the damaged myocardium.

This proposed mechanism has been suggested by other investigators examining stem cell therapy administered acutely post-MI. Angiogenic and cytoprotective growth factors such as VEGF and Ang-1 have been shown to significantly improve cardiac function through increased angiogenesis and decreases in infarct size when administered acutely or delivered as a gene construct post-MI (21, 57, 59). Similarly, Li et al. showed that increased capillary density following ASC treatment in a rat model of MI that correlated with higher VEGF mRNA and protein levels (57). Experiments have compared the effects of individual angiogenic genes, Ang-1 and VEGF with ASC therapy in a murine model of MI. While both Ang-1 and VEGF significantly improved cardiac performance, ASCs were superior in alleviating diastolic function and improving capillary density. In our present study, we observed that reduction in fibrosis following transplantation of preconditioned ASC was associated with increased angiogenesis in the infarcted areas in post-MI hearts as demonstrated by increased capillary and arteriole density within the infarct border zones (Fig. 20). Our results showed that the observed improved vascular
density after transplantation of preconditioned ASCs was correlated with increased secretion of growth factors with angiogenic potential; b-FGF, IGF-1 and VEGF (Chapter 2). While myocardial hypoxia during infarction has been demonstrated to induce expression of angiogenic growth factors including VEGF, the increased plasma levels of VEGF, Ang-1, and b-FGF we observed following treatment with preconditioned ASCs suggests the increased duration of ASCs within the ischemic microenvironment possibly allows them to function as “paracrine pumps” to sustain increased levels of growth factors which are known to lead to increased angiogenesis, suppression of cardiac fibrosis, and attenuation of cardiomyocyte apoptosis.

A number of studies have shown that apoptosis in cardiomyocytes contributes to the progression of heart failure after MI (97, 160), and chronic cardiac remodeling with chamber dilation and impaired systolic function is associated with increased myocyte apoptosis in the infarct border zone after MI (161). Our results demonstrate that transplantation of preconditioned ASCs abrogated resident cardiomyocyte apoptosis seen in non-treated ischemic hearts. Furthermore, the extent of apoptosis was significantly lower from preconditioned ASC treated hearts compared with control ASC treated hearts at 28 days post-MI. Overexpression of Ang-1, VEGF, or b-FGF has been shown to improve cell survival, neovascularization, and cardiac function by limiting the remodeling process in the scar while decreasing apoptosis of myocytes in the peri-infarct region (21, 58, 78). Transplantation of ASCs along with their secretion of VEGF, bFGF, and IGF attenuated cardiomyocyte apoptosis in vitro and in vivo (21). Preconditioning of ASCs through PDE-5 inhibition exerts a similar cardioprotective effect. The present results are consistent with the limitation of necrosis and apoptosis of adult cardiomyocytes treated with conditioned medium and preconditioned ASCs subjected to SI/RO
(Chapter 2) suggesting that the protective effect is attributed to increased duration and release of paracrine factors in the ischemic myocardium.

Besides the increased duration of pro-survival growth factors, another explanation for the cardioprotection seen following transplantation of preconditioned ASCs, could be due to the induction of NO generation following PDE-5 inhibition observed in Chapter 2. We speculate that increased duration of preconditioned ASCs concomitant with increased NO release could allow for diffusion of NO into the ischemic region and possibly preserve cardiomyocytes through the opening of mitoK\textsubscript{ATP} channels, preventing Ca\textsuperscript{2+} overload, and reducing oxidative stress. Moreover, we have shown that sildenafil attenuated apoptosis as well as necrosis in cardiomyocytes subjected to simulated ischemia and reoxygenation through nitric oxide (NO)-dependent pathway (75). It is also likely that the reduction of fibrotic tissue seen after cellular treatment with preconditioned ASCs could be the result of increased generation of NO that could then diffuse into the ischemic zone and restore the cGMP/PKG pathway. We have reported previously that sildenafil-induced increases in NO is involved in infarct size reduction after 24 h, even in a model of permanent LAD occlusion as the infarct sparing effect of sildenafil were blocked with NOS inhibitor, L-NAME (91). Although, there is no clear mechanism for the observed reduction of apoptosis and fibrosis following preconditioned ASC therapy, it is likely that increased release of NO along with pro-survival growth factors confers a cardioprotective mechanism through enhanced paracrine signaling. However, further studies are needed to investigate the role of NO in our model.

In conclusion, we have shown that \textit{ex vivo} preconditioning of ASCs by PDE-5 inhibition prior to transplantation into mice post-MI ameliorates LV remodeling, preserves LV function,
and reduced cardiomyocyte apoptosis and fibrosis possibly by improving stem cell survival and paracrine effects. Sildenafil is already being tested clinically in patients with heart failure and preserved ejection fraction (i.e. EF>50%) in the ongoing NIH multicenter trial (RELAX: Evaluating the Effectiveness of Sildenafil at Improving Health Outcomes and Exercise Ability in People With Diastolic Heart Failure; NCT00763867). We believe that the easy availability of ASCs from humans during surgery combined with the preconditioning strategy of blocking PDE-5 using clinically approved PDE-5 inhibitors may hold great promise for initiation of clinical trials in heart failure patients.

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Ischemic heart disease (IHD) remains a leading cause of morbidity and mortality despite recent advances in pharmacotherapy and improved acute care. Increased incidence of risk factors such as hypertension, obesity, diabetes, and smoking over the last several decades have all contributed to this increased prevalence of IHD. Therefore a conceptual breakthrough is needed to develop novel targets and strategies to prevent and cure IHD. In recent years, cardiac repair by stem cells has gained tremendous attention and imagination because of its initial success and safety potential. However, there are several major hurdles such as improvement in the number of reparative cells homing to injured areas, selection of ideal cell type(s), enhancement of regenerative and differentiation capacity of transplanted cells and optimization of extracellular milieu for engraftment and differentiation that would significantly impact long-term beneficial effects on cardiac functions. In the present study, we have used an innovative strategy of preconditioning the adipose stem cells (ASC) through inhibition of phosphodiesterase-5 (PDE-5) by sildenafil or genetic knockdown with a silencing vector. Our results showed that preconditioned ASC developed significant resistance against ischemic injury \textit{in vitro} with both of these approaches. Furthermore, transplantation of preconditioned ASC following a chronic
model of myocardial infarction (MI) resulted in amelioration of left ventricular (LV) remodeling, preserved LV function, reduced cardiomyocyte apoptosis and fibrosis possibly by improving stem cell survival through paracrine effects. We also like to mention that sildenafil is an interesting drug because it is widely used for treatment of erectile dysfunction and other cardiovascular disorders including pulmonary hypertension in patients. Moreover, there is an ongoing NIH multicenter trial (RELAX: Evaluating the Effectiveness of Sildenafil at Improving Health Outcomes and Exercise Ability in People With Diastolic Heart Failure; NCT00763867) in patients with heart failure. Another clinical trial on sildenafil has recently been initiated (REVERSE-DMD, NCT01168908) to treat Duchenne’s patients with cardiac disease, which is currently recruiting patients at the Johns Hopkins Medical Institutions. Because of the clinical safety and efficacy of sildenafil, we believe that this drug would be an ideal candidate to trigger cGMP-dependent survival pathway for preconditioning of ASC and their eventual use in patients for myocardial repair.

We also like to stress that despite our promising results, further studies need to be done to investigate the role of PDE-5 inhibition in enhancing stem cell therapy. In particular, the effect of preconditioned ASC transplantation should be examined in a model of ischemia/reperfusion injury model in addition to the permanent ischemia model used in the current investigation. This is because the majority of patients with acute myocardial infarction undergo spontaneous or therapeutic reperfusion. Reperfusion is known to cause tissue damage when blood supply returns to the tissue following ischemia. The restoration of circulation following the absence of oxygen and nutrients from blood results in a surge of free radicals in the form of reactive oxygen species and oxidative stress causing subsequent oxidative damage and inflammation.
Consequently, it is of utmost importance to determine whether ASC are effective when coronary occlusion is followed by reperfusion, an event that dramatically alters the milieu of the myocardial interstitium and of the myocardium itself.

In addition to the physiological studies, there is critical need to examine new molecular mechanisms by which preconditioning of ASC leads to improvement in cardiac function and preservation of myocardial structure. We will study the role of PKG in mediating the cytoprotective effect by using strategies such as overexpression of PKG-1α in ASC using adenoviral/lentiviral approaches prior to transplantation in the heart. We also plan to study the role frizzled related protein 2 (Sfrp2) as a survival molecule in ASCs preconditioned with PDE-5 inhibition/PKG1α overexpression. Recent studies have identified sfrp2 as a key survival molecule which acts via the canonical Wnt/β-catenin pathway (162). We anticipate that our current study and future efforts would lead to expanding the use of sildenafil and other clinically used PDE-5 inhibitors in regenerative medicine.
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<table>
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<tr>
<th>Degree</th>
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14. Varma A, Salloum FN, **Hoke NN**, Kukreja RC. Daily Therapy with the Phospholipase A2 Inhibitor, PX-18, Improves the Chronic Inflammatory State in Obese Mice and Reduces Infarct Size following Ischemia/Reperfusion. Accepted for presentation at the American Heart Association 2009 Annual Conference.

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Accepted for presentation at VCU’s Watt’s Day 2010 and Experimental Biology 2011 Annual Conference.