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Sildenafil Augments Early Protective Transcriptional Changes After Ischemia In Mouse Myocardium

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Abstract

Recently, targeting cyclic-GMP specific phosphodiesterase-5 (PDE5) has attracted much interest in several cardiopulmonary diseases, in particular myocardial ischemia (MI). Although multiple mechanisms were postulated for these beneficial effects at cellular level, early transcriptional changes were unknown. The aim of present study was to examine gene expression profiles in response to MI after 24h of ischemia in murine model and compare transcriptional modulation by sildenafil, a popular phosphodiesterase 5 (PDE5) inhibitor. Mice were divided into four groups: Control sham (C), Sildenafil sham (S), Control MI (CMI) and Sildenafil MI (SMI). Sildenafil was given at a dose of 0.7 mg/kg intraperitoneally 30 minutes before LAD occlusion. cDNA microarray analysis of peri-infarct tissue was done using a custom cloneset and employing a looped dye swap design. Replicate signals were median averaged and normalized using LOWESS algorithm. R/MAANOVA analysis was used and false discovery rate corrected permutation p-values < 0.005 were employed as significance thresholds. 156 genes were identified as significantly regulated demonstrating fold difference >1.5 in atleast one of the four groups. 52 genes were significantly upregulated in SMI compared to CMI. For a randomly chosen subset of genes (9), microarray data were confirmed through real time RT-PCR. The differentially expressed genes could be classified into following groups based on their function: Phosphorylation/dephosphorylation, Apoptosis, differentiation, ATP binding. Our results suggest that sildenafil treatment might regulate early genetic reprogramming strategy for preservation of the ischemic myocardium.

Keywords

Gene expression; myocardial infarction; microarray; sildenafil

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1. Introduction

Ischemic heart disease is the leading cause of mortality and morbidity in adults worldwide. The molecular mechanisms associated with myocardial infarction has been studied in animals extensively (Chandrasekar and Freeman, 1997; Chandrasekar et al., 1999; Maulik et al., 1999; Meissner et al., 2000; Kawata et al., 2001) and numerous cellular factors representing regulatory pathways (i.e. transcription factors, pro-inflammatory proteins, early response genes, growth and apoptosis factors and heat shock proteins) have been involved in episodes of ischemia. The development and usage of high-output gene microarrays have provided researchers a unique opportunity to perform global gene profiling and to study thousands of differentially expressed genes within different categories of genes, to attribute them to a specific condition and/or treatment. This technology enabled to study specific mechanisms involved in pathogenesis of many cardiovascular disorders. Johnatty et al (Johnatty et al., 2000) demonstrated that more than 100 genes were upregulated in the mechanically load-induced model of cardiac hypertrophy, where as, in a rat model of chronic myocardial infarction, Stanton et al. (Stanton et al., 2000) and Lyn et al. (Lyn et al., 2000) reported specific alterations of gene expression pattern in response to myocardial infarction. Even though several acute alterations were described in murine model of acute myocardial ischemia, changes in these alterations by pharmacotherapy has not been well documented.

Sildenafil is a potent inhibitor of phosphodiesterase 5 (PDE5), which belongs to large family of cyclic nucleotide phosphodiesterase enzymes that are differentially distributed in various tissues and its main action involves hydrolysis of cyclic GMP. Cellular levels of cGMP are regulated by the balance between synthesis by guanyl synthase and hydrolytic degradation by PDE5 in the myocardium. It has been shown that sildenafil, by increasing cellular concentration of cGMP and thereby activating various protein kinases, induces significant myocardial ischemic preconditioning leading to cardioprotection (Kukreja, 2006; Elrod et al., 2007; Salloum et al., 2007). Gillies et al (Gillies et al., 2002) demonstrated that sildenafil has significant vasomotor and hemodynamic effects which have potential to increase coronary blood flow and coronary flow reserve with little effect on heart rate, cardiac output and systemic and pulmonary vascular resistance in adults with ischemic heart disease. Another study (Ockaili et al., 2002) demonstrated, in animal models, that sildenafil exerts both early and delayed cardioprotective effects as evidenced by decrease in infarct size with significant, but mild decrease in systemic hemodynamics. They have demonstrated that involvement of mitoKATP channels plays an important role in mediating this protection. Zhang et al (Zhang et al., 2006) has further demonstrated that by increasing cGMP in the ischemic brain, sildenafil enhances neurogenesis and augments functional recovery in rats. It was also shown that sildenafil dilates coronary arteries, improves endothelial dysfunction and inhibits platelet activation with modest improvement in ischemic threshold in patients with coronary artery disease (Halcox et al., 2002).

Acute myocardial infarction induces ventricular remodeling, a process that can influence ventricular functions and survival outcomes (Pfeffer and Braunwald, 1990) and is directly implicated in postinfarction development of ventricular dilatation, leading to congestive heart failure. It has been postulated that viable regions of myocardium undergoes compensatory hypertrophy in a time-dependent manner resulting in re-conformation of ventricular architecture (Pfeffer and Braunwald, 1990). A rat model of myocardial infarction has been extensively studied to understand the functional, structural, and molecular changes associated with clinical ischemic heart disease (Pfeffer et al., 1979; Stanton et al., 2000). These led researchers to study substantial alterations in gene expression that eventually translate into profound changes in cells of the remodeling myocardium. Recently, the cDNA microarray technology has been productively employed to query >4,000 genes in response to permanent partial ligation of the coronary artery (Stanton et al., 2000). We have demonstrated earlier that

sildenafil induces angiogenic response in human coronary arteriolar endothelial cells through the expression of thioredoxin, heme oxygenase-1 and VEGF (Vidavalur et al., 2006). In addition we have shown recently that sildenafil induced neovascularization *in vivo* via upregulation of VEGF and angiopoietin-1 in ischemia-reperfused myocardium (Koneru et al., 2008).

In this study, we investigated the molecular mechanisms involved in pharmacotherapy with sildenafil leading to its cardioprotective effect by studying gene expression in mouse MI myocardium with cDNA microarray. The strategy of our present study was to observe the differential molecular profile of genes prior to ischemic and post ischemic insult in sildenafil treated animals.

2. Materials and Methods

2.1 Use of Animals

Twelve weeks old male C57BL/6 mice with mean body weight of 25 gm (range; 22 – 28 gm) were used for the study. All animals were maintained in accordance with the guidelines of the Animal care and Use Committee at University of Connecticut Health Center and National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Study Protocol

Viagra tablets (Pfizer Inc) were ground into powder and dissolved in 0.9% normal saline. This sildenafil solution was filtered (0.45μm pore size) before intraperitoneal injection. The control group received 0.2 ml of 0.9% normal saline alone. Sildenafil solution (0.7 mg/kg), equivalent to 50mg Viagra tablets used for patients with erectile dysfunction was used in the present study as described previously (Salloum et al., 2003). Normal saline (0.9%) for control or the Sildenafil solution (0.7 mg/kg) was given 30 minutes before myocardial infarction (MI).

Mice were anesthetized with 2,2,2-Tribromoethanol (Avertin), orally intubated with a 22G IV catheter, and ventilated with a rodent respirator (Harvard Apparatus). Hearts were exposed through the left lateral thoracotomy. MI was created by permanent left anterior descending coronary artery (LAD) ligation with 8-0 polypropylene sutures under a stereo zoom dissection microscope. The mice in the sham group underwent the same procedure except for the LAD ligation (Fig 1A). The lungs were inflated by positive end-expiratory pressure and the chest was closed with 6.0 nylon suture. These mice were randomized into 4 groups: control sham group (C), control MI group (CMI), sildenafil-treated sham group (S) and sildenafil-treated MI group (SMI).

Twenty four hours after MI, the hearts were excised and perfused with Krebs-Henseleit buffer through an aortic cannula to clear off the blood and to demarcate the risk area and the peri-infarct area. The peri-infarcted myocardial tissue sample was excised and rapidly snap-frozen in liquid nitrogen.

2.3 MicroArray protocol

2.3 (A) Informatics—A custom cDNA cloneset 6628 ESTs from the publicly available sequence verified clone sets, NIA (15K) and the BMAP (11K) containing non-overlapping GO or Locuslink annotated ESTs was printed in four non-adjacent replicates onto poly-Lysine coated substrates using 50% DMSO as a spotting solution for a spot diameter of 120 μm and a pitch of 170 μm. Printed arrays were then processed using a non aqueous processing protocol (Diehl et al., 2001).

The interpretation of data from microarray experiments is complicated by variation in fluorescent signal from array to array, differential hybridization efficiency and differential labeling efficiency for different sequences (Kerr and Churchill, 2001). In order to address these issues, multiple replicates from each gene-specific probe were spotted on each slide. All microarray hybridizations were performed in duplicate and each sample was labeled with both the red and the green dyes ("dye flip"). A "loop" design was used to establish the order of test and control samples co-hybridized on the different arrays (Fig. 1B) (Kerr and Churchill, 2001).

2.3 (B) cDNA synthesis, labeling and hybridization—Total RNA was converted into fluorescently labeled targets using T7 RNA polymerase in a single round linear amplification protocol (Van Gelder et al., 1990; Pabon et al., 2001). For each sample three separate reactions were performed with each 5 mg of total RNA and the respective cRNA pooled. Per channel 5 mg of the cRNA was then reverse transcribed in the presence of a random (8 mer) primer and amino allyl-modified dUTP) (Wang et al., 2003). After purification via Qiaquick column chromatography aminoallyl groups were reacted with the respective Alexa dye esters (555, 594 or 647) for 1 hr at RT in an argon environment. The fluorescently labeled cDNA was again purified via Qiaquick column chromatography, ethonal precipitated, dissolved in water, and the respective samples combined. After heat denaturation and adjustment to 1x hybridization, buffer samples were hybridized in a microfluidics based chamber (Biomicro Inc., Salt Lake City, UT) for 16 hours. Post hybridization the slides were washed with decreasing concentrations of SSC (2X -> 0.2X) at room temperature and spin dried AT 80×g for 2 minutes. cDNA labeling with Cye-dyes was repeated in reciprocal for each pair wise comparison in order to rule out difference calls resulting from differential labeling efficiency or fluorochrome luminescence.

2.3 (C) Detection and analysis—Raw images were acquired in an environmentally controlled room immediately after post hybridization processing of the slides using a *ScanArray* Express scanner (Perkin-Elmer Life And Analytical Sciences, Inc., Wellesley, MA). High-resolution, 16-bit, tagged image files were acquired after a series of fast, low resolution, exploratory scans to optimize high visual signal-to-noise ratios and signal linearity. All arrays were scanned at the same optimal laser power and gain. Raw signal and background intensities were extracted from the scanned images using the ImaGene software suite Version 5 (Biodiscovery Inc., El Segundo, CA)

2.4 Statistical Analysis

To minimize artifacts arising from low expression values, the genes with raw intensity values for Cy3 and Cy5>800 counts were chosen for differential analysis. For extraction of genes with expression levels affected by the treatment with Sildenafil, the raw data were funneled through a data preprocessing pipeline. Signal variability and sensitivity was controlled for by spotting each of the 6628 EST in quadruplicates and by performing the hybridization using a microfluidics hybridization chamber (Biomicro. Inc). The spotting in quadruplicates ensures an increased robustness towards slide imperfections of the signals observed. For data analysis the replicate signals were median averaged to minimize the effect of outliers and normalized using the LOWESS algorithm to remove intra slide dye bias. LOWESS normalized dataset were imputed using a pair wise random selection engine with the imputation permittence threshold set to maximal 50% of missing values. This preprocessed dataset was then analyzed using the R/MAANOVA package (Woo et al., 2005) with a fixed model and array, dye, and phenotype (treatment) as covariables. For selection of differentially expressed genes, false discovery rate corrected permutation p-values smaller than 0.005 were employed as significance thresholds. R/MAANOVA analysis was selected as the statistical method of choice since it permits the separate consideration of array and dye effects as experimental

covariables, omitting the requirement for interslide normalization approaches, notoriously known to introduce artificial bias (Woo et al., 2005).

Using this approach 156 genes were identified as significantly regulated among the 6628 ESTs queried in this study and these genes also demonstrated a fold difference greater than 1.5 in at least one of the four groups. 10 of the 156 genes were not annotated by either Locuslink or Entrez ID, leaving a total of 146 genes for further analysis by annotation.

2.5 Real time RT-PCR

RT was performed on 10ng total RNA isolated from left ventricular peri infarcted tissue of four groups. Real time RT-PCR was carried out using iCycler Iq detection system (Biorad, Hercules, CA) by SYBR Green 1 using β-actin as reference control. Primer sequences used for Real Time RT-PCR for nine genes tested were listed in table 6.

3. Results

3.1 Effects of MI on Myocardial Gene Expression

Using DNA microarrays, we identified 156 genes that were most likely differentially expressed between mice with untreated MI and sham after MI, there was induction of genes involved in inflammation, wound healing, DNA repair and extracellular matrix deposition. Heat shock proteins appear to be important in this process, with involvement of the alternative pathway indicated by induction of cell survival proteins. *HSP70 (6.5 fold)* and *HSP40 (5.6 fold)* genes were upregulated by more than 4 fold where as several protein kinases including *PRKWNK1 (-1.8 fold), AKAP9 (-1.79 fold)*, were down regulated. There was induction of genes coding for growth factors and proteins involved in steroid metabolism. Notable was the coordinate upregulation of genes involved in cytoskelatal rearrangement e.g.,*syndecan 4* and *dystonin* and anti apoptotic genes *BCL2L1(2 fold), Lipocalin (4 fold)* and *NFKBIA(1.7 fold)*. Finally, we detected robust induction of activation transcription factor 3 (*14.9 fold*) and beta-tranducin repeat containing protein (*10.6 fold*) (Table 1)

3. 2 Effects of Sildenafil on MI-Induced Gene Expression (SMI vs CMI)

When sildenafil treated MI hearts compared with control MI, sildenafil effectively induced more anti apoptotic, angiogenic and cytoskeleton remodeling genes (Table 3). Pronounced down regulation of interferon induced GTPASE *(-5.9 fold)* and homeobox b3 *(-2.5 fold)* genes were seen along with significant upregulation of *MAP3K6(2.8 fold), TIMP4 (2.5 fold), GADD45G (2.3 fold) and ESAM1 (2 fold)*. Similarly differential expression of genes between sildenafil sham (S) and sildenafil MI hearts (SMI), sildenafil sham(S) and control sham (C) were shown in Table 2 and 4 respectively. We have also analyzed the significantly affected genes between control and sildenafil groups (CMI/C vs SMI/S) as shown in Table 5

3.3 Chilibot analysis

It is now widely accepted that two forms of cell death, apoptosis and necrosis, occur in cardiomyocytes, and increasing evidence indicates that apoptosis plays an important role in the pathophysiology of cardiovascular diseases, especially myocardial ischemia. On the other hand, there has been renewed interest in agents and molecules to augment myocardial angiogenesis in ischemia/reperfusion setting, to preserve ischemic myocardium and improve functional recovery. In this study, we validated some of the important genes associated with these processes by Chilibot analysis (Chen and Sharp, 2004) which is designed for rapidly identifying relationships between genes, proteins, functional processes by searching *PubMed* literature database. The relationships are depicted in Fig.2. Adam15 and Notch4 were involved

in angiogenesis where as NFKBIA, MAP3K6, PYCARD, GADD45B were responsible for regulation of apoptosis.

3.4 Real time RT-PCR

Target validation by real time RT-PCR was done on randomly chosen 9 genes that were differentially expressed in microarray analysis. Results are presented in Figure 3. These results indicate that treatment of wild type mice with sildenafil induced up-regulation of angiogenic genes eg., *ADAM15 (1.5 fold)*, *NOTCH4 (1.7 fold)*, and anti-apoptotic genes e.g.,*NFKBIA (3 fold)*, *MAP3K6 (4 fold)*, *BCL2L1(1.4fold)*. Interestingly, sildenafil also down regulated pro apoptotic genes e.g., *PYCARD(0.5 fold)*, *TGTP (0.1fold)* in consistent with microarray results.

4. Discussion

This study explores for the first time how sildenafil treatment might affect the molecular profile of gene expression prior to ischemic injury and post ischemic insult in the murine model of myocardial ischemia. Relative gene expression changes in response to ischemia were determined using the expression profiles of time-matched control and sildenafil treated hearts as baseline. In response to ischemia, 156 genes showed significant altered expression (see Section 2.4 for statistical calculations).

High output gene expression profiling by microarray technologies has been successfully applied to study the transcriptional changes that occur in various tissues such as heart, vessels and blood cells in different cardiovascular disorders with the aim of unraveling the complex molecular pictures underlying human pathophysiology. Availability of animal models allowed the identification of early changes in gene expression before the onset of necrosis, particularly, in reversible myocardial ischemia and ischemia/reperfusion injury. In earlier experiments, transcriptome profiling studies performed during or following brief episodes of ischemia showed transcriptional reprogramming towards the activation of protective genes. In fact, ischemic areas of rat hearts subjected for 20 min to proximal coronary occlusion followed by 4 h of reperfusion, revealed upregulation of, growth factors, several heat shock proteins transcription factors, survival promoting molecules and anti-apoptotic factors (Simkhovich et al., 2003). In accordance with their findings, we found several genes in the present study which exhibited changes in expression in response to ischemia, i.e. heat shock proteins, *ATF3*, *GADD45G*, *MAP3K6*.

In contrast, a permanent coronary occlusion for over 24h resulting in myocardial infarction was shown to activate a different cardiac genetic program, clearly distinct from the protective program activated by reversible ischemia. The necrosis-induced program was characterized by overexpression of cardiac remodeling genes and apoptotic genes, and by underexpression of energy-generating pathways, such as that of fatty acid metabolism (Lyn et al., 2000; Sehl et al., 2000; Stanton et al., 2000; Jin et al., 2001). The global picture showed inflammatory and fibrotic repair responses within the necrotic myocardium and a compensatory hypertrophy of the remaining normal ventricle. Genes coding for extracellular matrix, cytoskeletal architecture, repair and remodeling processes showed upregulation while contractile proteins were downregulated,. The cardiac metabolic reprogramming was shown by the underexpression of several enzymes involved in the β-oxidation of the fatty acids pathway. Such deregulation indicates a change in the energy-generating processes in the injured heart, with reduced fatty acid utilization as energy source.

Another gene expression study conducted in mouse model 24 h following coronary occlusion reported a significantly increased expression of a mitogenic early growth response factor-1 and of its target gene *α-myosin heavy chain*, which could be considered as a protective response to ischemia (Lyn et al., 2000). Sehl et al. (Sehl et al., 2000) compared the expression profiles of

rat myocardium during development and after infarction in order to investigate the expression of fetal gene programs occurring in both models, heart failure caused by hypertension or by infarction, and the hypertrophy model (Chien et al., 1991; Boheler and Schwartz, 1992). The gene expression profile of the developing rat heart showed upregulation of signal transduction and growth regulatory proteins. Conversely, the expression profiling in response to infarction showed the activation of a damage-repair and remodeling processes, as reflected by overexpression of *osteopontin*, *fibronectin* and *collagen III*. Only a small group of genes such as natriuretic factors *ANP* and *BNP* showed similar expression profiles in both failure and hypertrophy models, whereas the large majority showed a discordant regulation. Thus, reversible ischemia appears to be associated with genetic reprogramming towards cardioprotection, whereas the response to necrosis is associated with altered repair and remodeling processes, metabolic reprogramming and enhanced apoptosis.

Our results indicate that there is a distinct pattern of mRNA expression in the murine myocardium following ischemia. An episode of 24 hours of ischemia significantly increased activating growth factor 3 (*ATF3*), Growth arrest and DNA-damage inducible 45 gamma and beta (*GADD45G*, *GADD45B*), heat shock protein 1b (*HSPA1B*) mRNAs. *ATF3* was shown to play an important role in growth regulation and importantly in the cellular process of apoptosis. More over, we have shown that several cell protective genes like *GADD45G*, *GADD45B*, *CXCL1*, *BTG2* are upregulated by myocardial ischemia apart from various stress stimuli (Liebermann and Hoffman, 2002). Our study confirm that several cardioprotective genes encoding heat shock proteins, especially HSP40 (*DNAJB1*) and HSP70 (*HSPA1B*) are overexpressed in peri infarct tissue after myocardial ischemia. Interestingly, in sildenafil treated sham after subjecting to ischemia for 24 hours, there is significant upregulation of TIMP4, which prevents or reduces the activity of metalloendopeptidases, enzymes that catalyze the hydrolysis of nonterminal peptide linkages in oligopeptides or polypeptides and survival promoting molecules like HSP70, along with redox regulating gene xanthine dehydrogenese (*Xdh*). Comparison between sildenafil treated ischemic myocardium and control ischemic myocardium revealed that sildenafil contributed to strong induction of anti apoptotic genes like *MAP3K6*, *BCL2L1* and cardio protective genes like *HIPK3*, *IGALS4*. Members of the Bcl-2 family of proteins are major regulators of mitochondrial cytochrome c release and downstream caspase activation and as such play an important role in the regulation of cardiomyocyte apoptosis (Chen et al., 2001; Kunisada et al., 2002; Sugioka et al., 2003). The family includes pro-apoptotic (e.g. *Bax and Bid*) and anti-apoptotic (e.g. *Bcl-2 and Bcl-xL*) members. Bcl-2 and Bcl-xL localize to the intracellular membranes of the mitochondria, ER and nuclear envelope. Overexpression of anti-apoptotic members of Bcl-2 family of proteins have previously been shown to protect cardiomyocytes from doxorubicin and hypoxic death (Mitogen-activated protein kinase kinase kinase (*MAP3K6*), a highly related serine/threonine kinase to Apoptosis signal-regulating kinase (ASK1) in the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase pathways, play multiple important roles in cytokine and stress responses (Takeda et al., 2007). By formation of functional heteromeric complex between different MAP3Ks may be advantageous for cells to cope with a wide variety of stimuli by fine regulation of cellular responses (Li et al., 2005).

On the other hand, sildenafil, induced significant changes in mRNA levels in sham animals. Genes involving anti-apoptosis, DNA repair, cell resistance to stress (*ATF3*, *GADD45G*, *BTRC*) are significantly upregulated (>10 fold), where as pro apoptotic genes like PYCARD, IGTP, TGTP are strongly down regulated. *TGTP* and *IGTP* belongs to GTPase superfamily whose functions are regulated by interferons (IFN) through the binding and hydrolysis of GTP. Among the $\approx 30^{+}$ functional categories of genes regulated by IFN, those that respond to IFN-αßγ include those involved in Ag presentation (class I MHC, $ß_2$-microglobulin, chaperone), cell death (Fas/FasL, TNFRI/II), inhibition of protein synthesis (IDO, tryptophanyl tRNA synthetase), adhesion (integrins, intercellular adhesion molecule, vascular cell adhesion

molecule) (Boehm et al., 1997; Carlow et al., 1998). *PYCARD*, also known as ASC (apoptosis-associated speck-like protein containing a CARD) is one putative component of the inflammasome and is involved, notably, in cell death triggered by stimuli that engage caspase-1, suggesting a coupling between the inflammatory and cell death pathways (Martinon et al., 2002; Mariathasan et al., 2004). More over, it has also been suggested that *PYCARD* is a mediator of NF-kappa B activation and Caspase-8-dependent apoptosis in *Ipaf* signaling pathway (Masumoto et al., 2003). Notch proteins function as receptors for membrane-bound ligands (Jagged and Delta-like) to regulate cell-fate determination. Sildenafil upregulated *Notch 4* expression in sham samples, by expressing an activated form of the *Notch4* gene specifically in endothelial cells. Uyttendaele et al demonstrated that Notch4 pathway is likely to play an intrinsic role in endothelial cells by regulating branching morphogenesis and patterning the developing vasculature (Uyttendaele et al., 2001).

In addition, sildenafil showed upregulation of some angiogenic genes e.g., *ADAMTS1*, *ADAM15*, *ESAM1* in peri infarcted myocardium. ADAM15 (named for a disintegrin and metalloprotease 15, metargidin) is a membrane-anchored glycoprotein that has been implicated in cell-cell or cell-matrix interactions and in the proteolysis of molecules on the cell surface or extracellular matrix. A high level of expression of ADAM15 was found in vascular cells, the endocardium, hypertrophic cells in developing bone, specific areas of the hippocampus and cerebellum. Horiuchi et al suggested that ADAM15 has an important role in mediating neovascularization in mice model of retinopathy of prematurity (Horiuchi et al., 2003).

In summary, our results show that sildenafil can inhibit some of the deleterious changes in gene expression that are induced by MI and augment a genetic reprogramming strategy for myocardial preservation. The data also indicate that some aspects of the pathophysiology are apparently unaffected by the treatment and these areas offer identification of factors that would induce cytoprotective genes or inhibit injury-related genes that could be the targets for novel therapeutic strategies.

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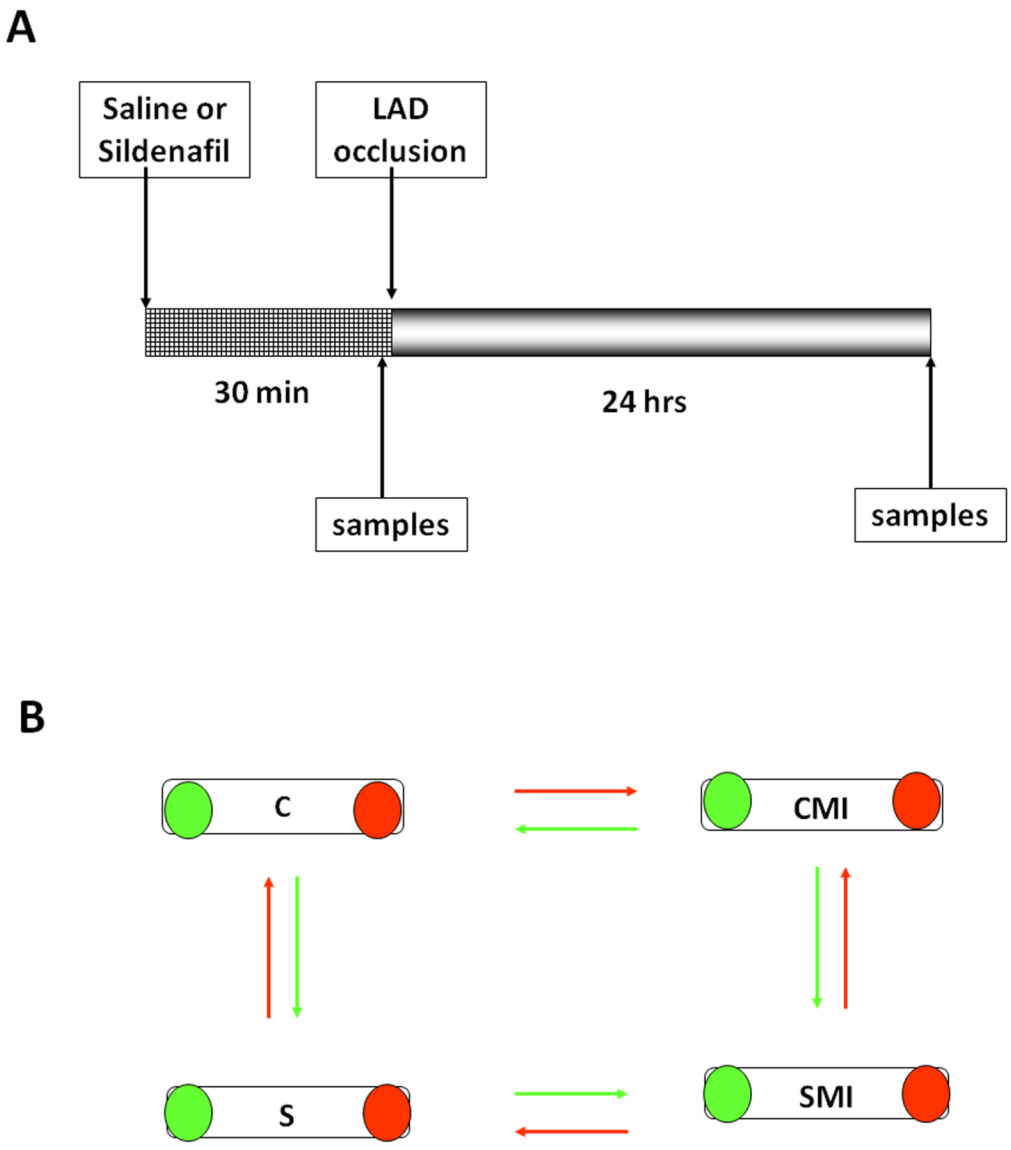

Fig 1.

A. Experimental protocol: Mice were randomly divided into 4 groups: (1) Control (C) (2) Control Myocardial infarction (CMI) (3) Sildenafil (S) and (4) Sildenafil MI (SMI). They were given either saline or sildenafil intraperitoneally at a dose of 0.7 mg/kg in treatment group 30 minutes before permanent LAD occlusion. The left ventricular tissues were collected for microarray analysis after 24 hours of MI (n = 6 in each group)

B. Experimental design: Loop design for gene expression analysis. Samples: C (Control or Sham), CMI (Control Myocardial Infaction), S (Sildenafil treated) and SMI (Sildenafil Myocardial Infarction). The red and green dots in each sample represent the “dye-flip” concept. Color-coded lines indicate the pairs of differentially labeled samples (red or green) co-hybridized on the different arrays.

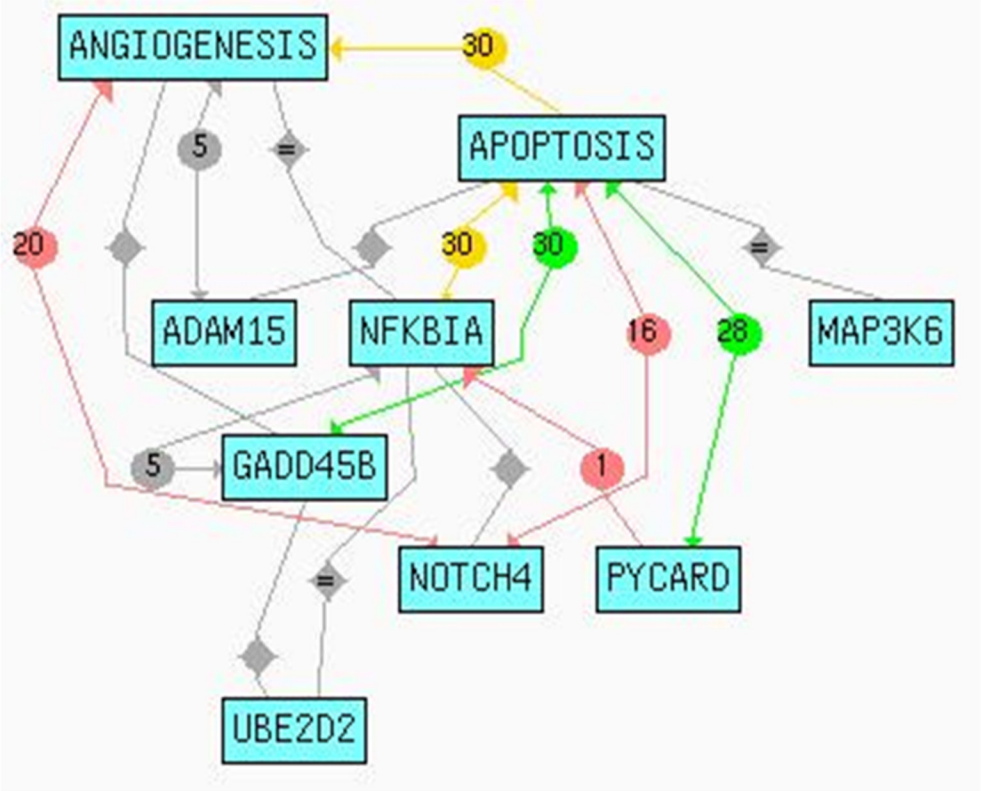

Fig 2. Chilibot text mining analysis

Chilibot text mining analysis of real time RT-PCR validated genes of known relationships between angiogenesis and statistically significant differentially expressed genes within enriched biological process terms of the GO analysis. Circles represent color coded interactive relationships: gray is neutral, green is stimulatory, red is inhibitory and yellow is both stimulatory and inhibitory. The numerical values within each icon present the number of PubMed abstracts used to validate the relationship (default is 50). Icons containing "_" represent parallel relationships, whereas gray rhomboidal icons indicate co-occurrence.

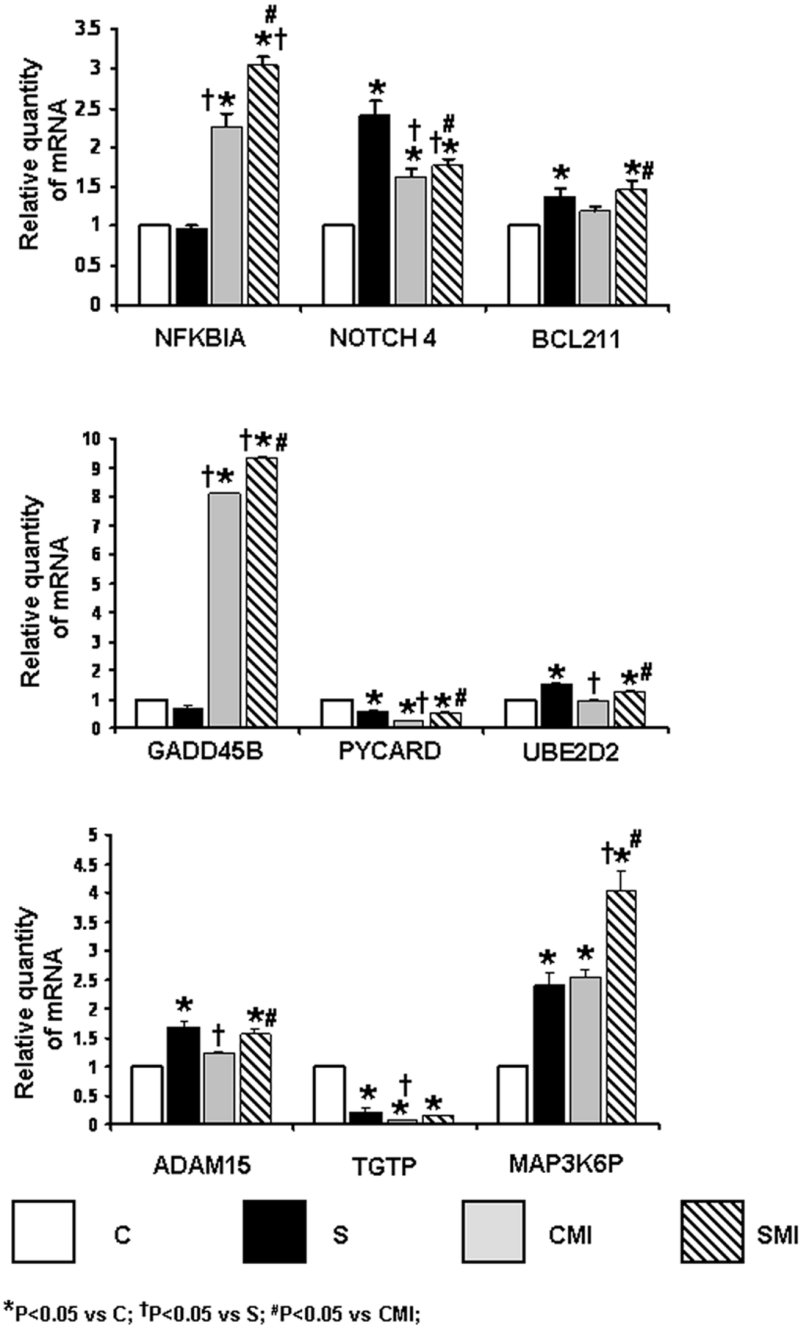

Fig 3. Confirmation of cDNA microarray data by real time RT-PCR

The relative mRNA expression in all four groups is shown for NFKBIA, GADD45B, ADAM15, NOTCH4, PYCARD, TGTP, BCL2L1, UBE2D2, MAP3K6P. The obtained data confirmed the cDNA microarray data in the transcriptional direction. (n = 6 animals for each group)

Table 1

Differential gene expression in control group after myocardial infarction (CMI/C)

Changes in Expression (CMI/C)	Gene Bank ID	Gene Name	Fold Change	Gene Symbol
Highly upregulated Fold Change >4	BG080433	activating transcription factor 3	14.91	ATF3
	BG080434	growth arrest and dna-damage-inducible 45 gamma	11.89	GADD45G
	BG080435	beta-transducin repeat containing protein	10.67	BTRC
	BG080436	chemokine (c-x-c motif) ligand 1	10.58	CXCL1
	BG080437	b-cell translocation gene 2, anti-proliferative	8.2	BTG2
	BG080438	isl1 transcription factor, lim/homeodomain (islet 1)	7.4	ISL1
	BG080439	growth arrest and dna-damage-inducible 45 beta	7.3	GADD45B
	BG080440	heat shock protein 1b	6.5	HSPA1B
	BG080441	dnaj (hsp40) homolog, subfamily b, member 1	5.6	DNAJB1
	BG080442	b-cell translocation gene 2, anti-proliferative	5.41	BTG2
	BG080443	jun-b oncogene	4.83	JUNB
	BG080444	a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1	4.8	ADAMTS 1
	BG080445	activity regulated cytoskeletal-associated protein	4.74	ARC
	BG080446	s100 calcium binding protein a9 (calgranulin b)	4.31	S100A9
	BG080447	lipocalin 2	4.05	LCN2
Highly downregulated Fold Change > -1.5	BG083533	cell division cycle 2-like 5 (cholinesterase-related cell division controller)	-1.6	CDC2L5
	AI840919	dna-damage inducible transcript 3	-1.64	DDIT3
	BG079603	a kinase (prka) anchor protein (yotiao) 9	-1.79	AKAP9
	BG080117	protein kinase, lysine deficient 1	-1.83	PRKWNK1
	BG082511	golgi autoantigen, golgin subfamily a, 4	-1.87	GOLGA4
	BG077753	sar1a gene homolog 1 (s. cerevisiae)	-1.98	SARA1
	BG076444	eukaryotic translation initiation factor 3, subunit 10 (theta)	-1.98	EIF3S10
	BG078030	keratin complex 1, acidic, gene 19	-2.46	KRT1-19
	CK335203	poly (a) polymerase alpha	-2.55	PAPOLA

Table 2

Differential gene expression in sildenafil treated group after myocardial infarction (SMI/S)

Changes in Expression (SMI/S)	Gene Bank ID	Gene Name	Fold Change	Gene Symbol
Highly upregulated Fold Change >1.5	AI835088	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	1.85	HERPUD1
	BG084374	tissue inhibitor of metalloproteinase 4	1.74	TIMP4
	BG088321	xanthine dehydrogenase	1.62	XDH
	AI841289	heat shock protein 1b	1.61	HSPA1B
	AI853669	mcf.2 transforming sequence-like	1.52	MCF2L
	BG087558	histocompatibility 2, d region locus 1	1.5	H2-D1
Highly downregulated Fold Change > -1.25	BG078934	eukaryotic translation initiation factor 1a	-1.28	EIFLA
	BG080268	chemokine (c-x-c motif) ligand 1	-1.28	CXCL1
	AI851187	ecotropic viral integration site 2a	-1.29	EVI2A
	AI845893	isl1 transcription factor, lim/homeodomain (islet 1)	-1.3	ISL1
	BG087429	complement component 3	-1.32	C3
	BG077753	sar1a gene homolog 1 (s. cerevisiae)	-1.32	SARA1
	BG080418	c-type lectin domain family 4, member e	-1.34	CLEC4E
	AI838607	thrombospondin 1	-1.39	THBS1
	BG083627	antizyme inhibitor 1	-1.4	AZIN1
	BG083332	lactotransferrin	-1.45	LTF
	BG081493	selectin, platelet	-1.5	SELP
	AI845405	spastic paraplegia 4 homolog (human)	-1.9	SPG4
	BG084937	aryl hydrocarbon receptor nuclear translocator-like	-3.69	ARNTL

Table 3

Differential gene expression between sildenafil treated myocardial infarction (SMI) and control myocardial infarction (CMI) groups

Changes in Expression (SMI/CMI)	Gene Bank ID	Gene Name	Fold Change	Gene Symbol
Highly upregulated Fold Change > 1.5	AI847285	mitogen-activated protein kinase kinase kinase 6	2.81	MAP3K6
	BG084374	tissue inhibitor of metalloproteinase 4	2.58	TIMP4
	BG080485	growth arrest and dna-damage-inducible 45 gamma	2.30	GADD45G
	BG079002	lectin, galactose binding, soluble 4	2.26	LGALS4
	BG077536	endothelial cell-specific adhesion molecule	2.06	ESAM1
	AI848824	dnaj (hsp40) homolog, subfamily b, member 5	1.79	DNAJB5
	AI844575	potassium voltage-gated channel, shaker-related subfamily, member 5	1.76	KCNA5
	BG069628	immediate early response 5	1.76	IER5
	BG082503	cbp/p300-interacting transactivator, with glu/asp-carboxy-terminal domain, 4	1.74	CITED4
	BG080836	homeodomain interacting protein kinase 3	1.73	HIPK3
	AI840919	dna-damage inducible transcript 3	1.73	DDIT3
	BG066495	dnaj (hsp40) homolog, subfamily b, member 1	1.61	DNAJB1
	AI844365	bcl2-like 1	1.58	BCL2L1
	BG080666	immediate early response 3	1.53	IER3
Higly downregulated Fold Change > -3	BG088898	glycoprotein 49 a	-2.56	GP49A
	BG086115	homeo box b3	-2.59	HOXB3
	AI854552	interferon-induced protein with tetratricopeptide repeats 3	-2.67	IFIT3
	AI840024	apolipoprotein d	-2.68	APOD
	CK335051	zinc finger protein 62	-2.81	ZFP62
	BG085588	s100 calcium binding protein a9 (calgranulin b)	-3.51	S100A9
	BG080979	olfactory receptor 56	-3.62	OLFR56
	BG070106	lipocalin 2	-3.64	LCN2
	BG088770	interferon gamma induced gtpase	-4.81	IGTP
	AI838580	interferon gamma induced gtpase	-5.91	IGTP

Table 4

Differential gene expression between sildenafil treated sham (S) and control sham (C) groups

Changes in Expression (S/C)	Gene Bank ID	Gene Name	Fold Change	Gene Symbol
Highly upregulated Fold Change > 5	BG080485	growth arrest and dna-damage-inducible 45 gamma	17.33	GADD45G
	BG087065	beta-transducin repeat containing protein	16.22	BTRC
	BG080433	activating transcription factor 3	10.96	ATF3
	AI845893	isl1 transcription factor, lim/homeodomain (islet 1)	9.72	ISL1
	BG080268	chemokine (c-x-c motif) ligand 1	9.03	CXCL1
	AI848850	growth arrest and dna-damage-inducible 45 beta	7.87	GADD45B
	BG078965	a disintegrin-like and metalloprotease with thrombospondin type 1 motif	7.82	ADAMTS1
	BG077422	dnaj (hsp40) homolog, subfamily b, member 1	7.38	BCAP31
	AI850555	jun-b oncogene	6.83	JUNB
	AI838607	thrombospondin 1	5.36	THBS1
	AI836864	forkhead box g1	5.15	FOXG1
	AI851775	b-cell translocation gene 2, anti-proliferative	5.14	BTG2
Highly downregulated Fold Change > -2.5	BG084904	pyd and card domain containing	-2.57	PYCARD
	BG080979	olfactory receptor 56	-3.89	OLFR56
	AI854552	interferon-induced protein with tetratricopeptide repeats 3	-3.91	IFIT3
	AI838580	interferon gamma induced gtpase	-6.16	IGTP
	BG088770	interferon gamma induced gtpase	-10.78	IGTP

Table 5

Differential gene expression between sildenfil (SMI/S) versus control (CMI/C) groups

Changes in Expression	Gene Bank ID	Gene Name	Fold Change	Gene Symbol
Highly upregulated Fold Change >2	BG084374	tissue inhibitor of metalloproteinase 4	2.84	TIMP4
	AI847285	mitogen-activated protein kinase kinase kinase 6	2.57	MAP3K6
	AI853669	mcf.2 transforming sequence-like	2.47	MCF2L
	CK335203	poly (a) polymerase alpha	2.36	PAPOLA
	BG080836	homeodomain interacting protein kinase 3	2.14	HIPK3
	BG080820	dnaj (hsp40) homolog, subfamily b, member 4	2.13	DNAJB4
	AI836767	potassium inwardly-rectifying channel, subfamily j, member 4	2.08	KCNJ4
	BG079002	lectin, galactose binding, soluble 4	2.06	LGALS4
	BG080672	interferon-related developmental regulator 1	2.00	IFRD1
Highly downregulated Fold Change > -2.5	BG085588	s100 calcium binding protein a9 (calgranulin b)	-2.52	S100A9
	BG083864	selectin, platelet (p-selectin) ligand	-2.55	SELPL
	AI845967	cathepsin s	-2.65	CTSS
	AI846176	riken cdna a930015d03 gene	-2.75	A930015D03RIK
	BG088898	glycoprotein 49 a	-2.77	GP49A
	BG084118	carbonyl reductase 2	-2.88	CBR2
	AI854552	interferon-induced protein with tetratricopeptide repeats 3	-2.89	IFIT3
	AI851187	ecotropic viral integration site 2a	-3.27	EVI2A
	BG070106	lipocalin 2	-3.39	LCN2
	CK335051	zinc finger protein 62	-3.49	ZFP62
	BG087868	complement component 1, q subcomponent, beta polypeptide	-3.55	C1QB
	BG080979	olfactory receptor 56	-3.61	OLFR56
	BG086115	homeo box b3	-3.93	HOXB3
	AI838580	interferon gamma induced gtpase	-4.22	IGTP

Table 6

Primer pairs designed for RT-PCR

Gene	Forward Primer	Reverse Primer
Pycard	5′- aaaagttcaagatgaagctgctg -3′	5′- ctcctgtaagcccatgtctctaa -3′
Ubed2	5′- caatccagatgatcctttagtgc -3′	5′- acatcgcatacttctgagtccat -3′
Gadd45b	5′- atatcgctctgcagattcacttc -3′	5′- tctgtatgacagttcgtgaccag -3′
Tgtp	5′- ccaccattaactgcaagagagat -3′	5′- gaagtcatccacagacatgttca -3′
Nfkbia	5′- tttgggtgctgatgtcaacgctca -3′	5′- agcgaaaccaggtcaggattctgc -3′
Bcl2l1	5′- ggagtaaactggggtcgcatcgt -3′	5′- acctgcatctccttgtctacgctt -3′
Notch4	5′- ggcacctgccagaacacagct -3′	5′- gcacagtcatccaggttctcctca -3′
Map3k6	5′-cgctgccttagttatggggacacc -3′	5′- cggcgcttgctctcctggtg -3′
Adam15	5′- gattgtggatgataattcagagg -3′	5′- gctcatttctatcaggttgtgct -3′