Phosphodiesterase-5 Inhibitor Sildenafil Preconditions Adult Cardiac Myocytes against Necrosis and Apoptosis

ESSENTIAL ROLE OF NITRIC OXIDE SIGNALING*

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We investigated the effect of sildenafil in protection against necrosis or apoptosis in cardiomyocytes. Adult mouse ventricular myocytes were treated with sildenafil (1 or 10 μM) for 1 h before 40 min of simulated ischemia (SI). Necrosis was determined by trypan blue exclusion and lactate dehydrogenase release following SI alone or plus 18 h of reoxygenation (RO). Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated nick end labeling assay and mitochondrial membrane potential measured using a fluorescent probe 5',5',6',6'-tetrachloro-1',3',3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). Sildenafil reduced necrosis as indicated by decrease in trypan blue-positive myocytes and leakage of lactate dehydrogenase compared with untreated cells after either SI or SI-RO. The number of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive myocytes or loss of JC-1 fluorescence following SI and 18 h of RO was attenuated in the sildenafil-treated group with concomitant inhibition of caspase 3 activity. An early increase in Bel-2 to Bax ratio with sildenafil treatment was also observed in myocytes after SI-RO. The increase of Bel-2 expression by sildenafil was inhibited by nitric-oxide synthase (NOS) inhibitor, l-nitro-arginine-methyl-ester. The drug also enhanced mRNA and protein content of inducible NOS (iNOS) and endothelial NOS (eNOS) in the myocytes. Sildenafil-induced protection against necrosis and apoptosis was absent in the myocytes derived from iNOS knock-out mice and was attenuated in eNOS knock-out myocytes. The up-regulation of Bel-2 expression by sildenafil was also absent in iNOS-deficient myocytes. Reverse transcription-PCR, Western blots, and immunohistochemical assay confirmed the expression of phosphodiesterase-5 in mouse cardiomyocytes. These data provide strong evidence for a direct protective effect of sildenafil against necrosis and apoptosis through NO signaling pathway. The results may have possible therapeutic potential in preventing myocyte cell death following ischemia/reperfusion.

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Sildenafil citrate is a selective inhibitor of phosphodiesterase-5 (PDE5)1 that catalyzes the breakdown of cGMP, one of the primary factors causing smooth muscle relaxation. By means of its potent action of enhancing NO-driven cGMP accumulation and ensuing vasodilation in corpus cavernosum, sildenafil has become the most widely used drug for treating erectile dysfunction in men since its market debut under the trade name Viagra® in 1998 (1–3). In recent years, there are several studies on sildenafil for its therapeutic applications in diseases other than erectile dysfunction (4–11). For example, sildenafil has been shown to enhance flow-mediated vasodilation in chronic heart failure patients (4). Because of its potent vasodilatory action, sildenafil has also been extensively studied for treating primary or hypoxia-induced pulmonary hypertension in adults and children (5–7, 9, 10). An epidemiological study (12) suggested that patients receiving sildenafil therapy for erectile dysfunction had reduced incidence of myocardial infarction than the general population matched with age, gender, and other risk factors. More interestingly, our laboratory recently discovered a powerful preconditioning-like effect of sildenafil in rabbit hearts (13). Either intravenous or oral administration of sildenafil caused significant reduction of infarct size following ischemia/reperfusion in the myocardium. This protection was blocked by 5-hydroxydecanoate, a selective blocker of mitochondrial ATP-sensitive potassium channels. Sildenafil-induced cardioprotection has also been demonstrated in a model of global ischemia and reperfusion in mouse (14) as well as rat (15). In addition, a recent study in rabbits has shown that early translocation of protein kinase C, especially the ρ, 4, and δ isofoms to the membranous fractions, may play an essential role in sildenafil-induced cardioprotection (16). Based on these studies (13–16), we postulated that sildenafil triggers signaling cascade that involves the activation of protein kinase C, the generation of NO, and the accumulation of cGMP in the myocardium through inducible and endothelial nitric-oxide synthase (iNOS and eNOS), thereby leading to cardioprotection via opening of mitochondrial ATP-sensitive potassium channels (17–19).

Despite these novel and exciting observations, several fundamental issues concerning the mechanisms of sildenafil-induced cardioprotection still need to be addressed. First, it is possible that the profound vasodilation caused by sildenafil may potentially trigger a preconditioning response in the...
heart. However, it remains unknown whether the drug also exerts a direct protective effect in the cardiomyocytes independent of its vascular/hypotensive effect. Second, although studies in intact hearts have demonstrated a significant protective effect of sildenafil against necrosis (infarction), there is absolutely no information in the literature suggesting that the drug also inhibits apoptosis or whether the mitochondria are the targets of protection in the cardiomyocytes. Third, despite the fact that NO signaling plays an important role in sildenafil-induced delayed preconditioning in the intact mouse heart, it is not known whether this pathway also regulates necrosis or apoptosis in the cardiomyocytes following simulated ischemia (SI) and reoxygenation (RO). Fourth, there is controversy over the presence of PDE5 in the mammalian ventricular cardiomyocytes (20–23), which may serve as the primary target for the action of sildenafil. To address these critical issues, we designed the current investigation to demonstrate the direct protective effect of sildenafil in a cardiomyocytes model of SI-RO injury. This model was particularly useful in studying the protective effect of sildenafil independent of any vascular effects or other cell types. We determined the effect of eNOS/iNOS in necrosis and apoptosis using cardiomyocytes derived from gene knock-out mice. Moreover, this study allowed us to directly knock-out PDE5 expression in ventricular cardiomyocytes.

EXPERIMENTAL PROCEDURES

Isolation of Ventricular Cardiomyocytes—Adult male outbred ICR mice were purchased from Harlan (Indianapolis, IN). The adult male iNOS and eNOS knock-out mice (stock numbers 002596 and 002684) and their corresponding wild-type controls (B6,129 and C57BL/6j) were supplied by The Jackson Laboratory (Bar Harbor, ME). 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 (24, 25). In brief, the mouse was anesthetized with pentobarbital sodium (100 mg/kg intraperitoneally), and the heart was quickly removed from the chest. Within 3 min, the aortic opening was cannulated onto a Langendorff perfusion system (26), and the heart was retrogradely perfused (37 °C) at a constant pressure of 56 mm Hg for 15 min with Ca2+/Mg2+-free Tyrode’s solution and then perfused with a Ca2+-based buffer containing 120 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO4, 1.2 mM NaH2PO4, 5.6 mM glucose, 20 mM NaHCO3, 10 mM 2,3-butanedione monoxime, and 5 mM taurine that was continuously gassed with 95% O2 and 5% CO2. RO was accomplished by replacing the ischemic buffer with normal medium under normoxic conditions. Assessment of cell necrosis and apoptosis was performed at two time points of RO, i.e. 1 and 18 h.

Evaluation of Cell Viability—Cell viability was assessed by trypan blue exclusion assay and LDH release in the medium. At the end of protocol, 20 μl of 0.4% trypan blue (Sigma-Aldrich) was added into the culture dish. After ~5 min of equilibration, the cells were counted under microscope. For LDH measurements, the cellular medium was collected, and the enzyme activity was monitored spectrophotometrically using an assay kit (Sigma-Aldrich).

TUNEL Staining and Measurement of Mitochondrial Membrane Potential—Cardiomyocyte apoptosis was analyzed by TUNEL staining, using a kit purchased from BD Biosciences that detects nuclear DNA fragmentation via a fluorescence assay as previously reported (28). In brief, after SI and 18 h of RO, the cells in two chamber slides were fixed with 4% formaldehyde/phosphate-buffered saline at 4 °C for 25 min and subjected to TUNEL assay according to the manufacturer’s protocol. Two slides were then counterstained with Vectashield mounting medium with 4’,6-diamidino-2-phenylindole (a DNA intercalating dye for visualizing nuclei in fixed cells; catalogue number H-1200, Vector Laboratories). Additionally, cardiomyocyte apoptosis was detected with a mitochondrial membrane potential (Δψm) detection kit provided by Becton. The cells were stained with a dye, 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl-carboyanine iodide (JC-1), at 37 °C for 15 min, and then washed with 5% CO2 incubator and rinsed with assay buffer according to the manufacturer’s protocol. The stained cells were examined under an Olympus IX70 fluorescence microscope.

Activated Caspase 3 Detection—Active caspase was detected using the Caspase™ Caspase 3,7 in situ assay kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions. In this assay, the cell-permeable noncytotoxic fluorochrome inhibitors of caspases binds covalently to a reactive cysteine residue on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. This kit uses a carboxyfluorescein labeled fluoromet ketone peptide inhibitor of caspases-3 and -7 (SR-DEVDFMK), which emits a red fluorescence. The red fluorescent signal is a direct measure of the amount of active caspase 3 in the cell at the time the reagent was added. The stained cells were immediately examined under an Nikon epi-fluorescence microscope using a band pass filter (excitation, 550 nm; emission, >580 nm) to view the red fluorescence of active caspase-positive cells. Hoechst stain was detected using a UV filter with excitation at 365 and emission at 480 nm.

RT-PCR of iNOS, eNOS, and PDE5—The total RNA was isolated from untreated and sildenafil-treated cardiomyocytes using TRI reagent (Molecular Research Center) as described previously (14). The RNA was purified and then reverse transcribed for RT reactions using a OneStep RT-PCR kit from Qiagen. The oligonucleotide primers were synthesized by Integrated DNA technology, according to the published sequences for mouse iNOS (29), eNOS (30), and β-actin (28), which served as an internal control. The transcript levels of iNOS and eNOS were quantified by real time PCR performed in the ABI prism 7900HT sequence detection system. For each sample, 5 μl of the 1:20 dilution of the TaqMan One Step PCR Master Mix reagent kit (Product 4309169). All of the samples were processed in triplicate according to the manufacturer’s recommended conditions. The cycling conditions were: 48 °C for 30 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The cycle threshold was determined to provide the optimal standard curve values (0.98–1.0). The probes and primers (Table I) were designed by the Nucleic Acid Research Facilities of Virginia Commonwealth University, using Primer Express 2.0. The probes were labeled in the 5′ end with 6-carboxyfluorescein and in the 3′ end with 6-carboxytetramethylrhodamine. Eukaryotic 18 S rRNA TaqMan endogenous control (Product 4310893E; Applied Biosystems) was used to normalize the transcript levels. The following amounts of RNA was used for real time PCR: 20 ng for iNOS, 4 ng for eNOS, and 1 ng for 18 S rRNA.

PDE5 was amplified by using the primers based on the mouse PDE5A cDNA sequence (GenBank™ accession number NM_153422; also see Table I). The RT-PCR products were electrophoresed on 2% Tris-acetate-EDTA-agarose gel.

Western blots for Bax, Bcl-2, iNOS, eNOS, and PDE5—Total soluble protein was extracted from the cells with reporters lysis buffer (Pro-mega). The homogenate was centrifuged at 10,000 × g for 5 min under 4 °C, and the supernatant was recovered. 25 μg of protein from each sample was separated by 12% acrylamide gels and transferred to nitrocellulose membrane and then blocked with 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.1% Tween 20) for 1 h. The membrane was then incubated with rabbit polyclonal primary antibody at a dilution of 1:1000 for each of the respective proteins, i.e. Bax, Bcl-2,
Table 1

| Primer/probe sequences | Primer/probe sequences |
|------------------------|------------------------|
| iNOS                   | eNOS                   |
| Forward: CAGAGAGGAAAGACACTCCTCATATGG | Forward: TGACCCTGAAAACCCCTACG |
| Reverse: TGACACACAGGAAACACTCCTCCTCATATGG | Reverse: TACACGTGAAAACCCCTACG |
| eNOS                   | PDE5A                  |
| Forward: CACCAAGGAAACCACACCTTTTTACAA | Forward: AACGACACCCCGCTGACAC |
| Reverse: CACACCTGCCATCACACGC | Reverse: GCCACCAGCTGACAC |

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iNOS, eNOS (Santa Cruz), or PDE5 (Calbiochem) for 2 h before being washed and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000; Amersham Biosciences) for 1 h. The blots were developed using a chemiluminescent system. The optical density for each band was scanned and quantified using the identical densitometric system described above.

Immunohistochemistry of PDE5—Localization of PDE5A protein in cardiomyocytes was performed according to the method of Senzaki et al. (23). The cardiomyocytes were fixed in 4% paraformaldehyde and then treated with 0.1% sodium citrate and 0.1% Triton X-100. The cells were then preincubated with normal donkey antiserum for 30 min and then incubated overnight at 4 °C with polyclonal rabbit PDE5A antibody (Calbiochem) at 1:10,000 dilution. Incubation with secondary antibody was performed at room temperature for 1 h with anti-rabbit Alexa 488 (Molecular Probes). Imaging was performed on a Zeiss LSM 510 META confocal laser scanning microscope.

Data Analysis and Statistics—The data are presented as the means ± S.E. The difference between control and sildenafil-treated groups or among the multiple treatment groups was analyzed respectively with unpaired t test or one-way analysis of variance followed by Student-Newman-Keuls post-hoc test. $p < 0.05$ was considered to be statistically significant.

Results

Effect of Sildenafil on Cardiomyocyte Necrosis—Our method for cell preparations yielded at least 70% of the cardiomyocytes with rod shape morphology, which was similar to previously reported studies (24, 25). Fig. 1A shows a typical preparation of isolated adult mouse cardiomyocytes used in the present studies. After 40 min of SI, the percentage of trypan blue positive cardiomyocytes increased with respect to control cardiomyocytes, i.e. from 3 ± 0.2% of total counted cells in the control group to 35 ± 1% in the SI group, $p < 0.05$. Pretreatment with sildenafil for 1 h resulted in the decrease in trypan blue positive cardiomyocytes, i.e. from 35 ± 1% of the total counted cells in the untreated SI group to 17 ± 0.5 and 18 ± 1% in 1 and 10 μM sildenafil-treated groups, respectively ($n = 3$; $p < 0.05$, Fig. 1D). After 40 min of SI and 1 h or 18 h of RO, the trypan blue positive cardiomyocytes further increased to 40 ± 2 and 54 ± 1%, respectively. Again, prior treatment with sildenafil reduced the trypan blue-positive cells as compared with the untreated SI-RO group ($p < 0.001$, $n = 3$; Fig. 1, B–D). Similar results were obtained by measurement of LDH release in the medium. As shown in Fig. 1E, LDH increased in the medium following SI and SI-RO (1 or 18 h), which is attenuated by prior treatment with sildenafil. Also, the attenuation in LDH release was independent of the dose of sildenafil used in these experiments.

Effect of Sildenafil on Cardiomyocyte Apoptosis—Despite significant necrosis following 40 min of SI and 1 h of RO (Fig. 1, D and E), apoptosis was not detectable under these conditions (data not shown). However, apoptotic cell death became clearly evident following 40 min of SI and 18 h of RO, i.e. 24.7 ± 1.2% of TUNEL-positive cells ($p < 0.001$ versus nonischemic control, $n = 4$). The number of TUNEL-positive cells were reduced to 3.5 ± 0.6% in cardiomyocytes treated with 10 μM sildenafil ($p < 0.001$ versus SI-RO and $p < 0.05$ versus untreated nonischemic control cardiomyocytes, $n = 4$; Fig. 2, A–I). The intense red fluorescence of active caspase was clearly observed in myocytes following 40 min of SI and 18 h of RO (Fig. 3B). But the red fluorescence decreased significantly in myocytes treated with 1 or 10 μM sildenafil before 40 min of SI and 18 h of RO (Fig. 3, C and D).

Effect of Sildenafil on Mitochondrial Membrane Potential—It has been demonstrated that components of ischemia activate the mitochondrial death pathway in cardiac myocytes (31). In the present study, we used cationic lipophilic probe JC-1 (32–34) to assess the mitochondrial membrane potential ($\Delta \psi_m$). In this assay, the mitochondria of nonapoptotic cells appeared in red following the aggregation of the JC-1 reagent, which emits red fluorescence at 590 nm (Fig. 4A). In contrast, in the apoptotic or dead cells the JC-1 dye remained in its monomeric form, thereby emitting relatively more green fluorescence (Fig. 4C). For positive control, cardiomyocytes were treated with an uncoupler of mitochondrial respiration, carbonyl cyanide m-chlorophenylhydrazone (10 μM), for 15 min before the initiation of JC-1 dye loading. As shown in Fig. 4B, there was a dissociation of JC-1 aggregates in the mitochondrial as a result of drop in $\Delta \psi_m$. Consequently, intense green fluorescence was observed in the presence of carbonyl cyanide m-chlorophenylhydrazone in these cells. A reduction in the ratio of red to green fluorescence indicates a fall in $\Delta \psi_m$. In the cardiomyocytes subjected to SI and RO for 18 h, the red aggregated fluorescence decreased, and the monomer green fluorescence increased (Fig. 4C). Quantitative measurements showed that SI-RO cardiomyocytes had a lower JC-1 aggregate/monomer ratio as compared with the control nonischemic cells (i.e. 16.8 ± 0.5 versus 110.7 ± 0.7, p < 0.001, n = 3; Fig. 4E). The JC-1 aggregate/monomer ratio was increased significantly to 34.9 ± 0.5 in cardiomyocytes treated with 10 μM sildenafil (Fig. 4, D and E).

Effect of Sildenafil on Bcl-2 Expression: Role of NO Signaling—We further examined the effect of sildenafil on expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein, Bax in the cardiomyocytes. As shown in Fig. 5 (A–C), sildenafil enhanced the expression of Bcl-2 in a time-dependent manner. The robust induction of Bcl-2 occurred in 10 μM sildenafil-pretreated myocytes after 40 min of SI and 1 h of RO but disappeared after 18 h of RO. When normalized with the expression of β-actin, there was a significant increase in Bcl-2 to Bax ratio following 1 h of RO in sildenafil-treated cardiomyocytes (Fig. 5C). This early induction of Bcl-2 by sildenafil could be responsible for its anti-apoptotic effect in the present study (Fig. 2).

To demonstrate a possible link between NO signaling and induction of Bcl-2 by sildenafil, we co-incubated cardiomyocytes with 100 μM of nonselective NOS inhibitor l-NAME with 10 μM sildenafil prior to SI-RO. The results show that sildenafil-induced Bcl-2 up-regulation was completely abolished by l-NAME, suggesting a NOS-dependent anti-apoptotic effect of sildenafil in cardiomyocytes (Fig. 5, D and E).

Effect of Sildenafil on iNOS and eNOS Expression in Cardiomyocytes—An increase in mRNA of iNOS and eNOS (to a lesser extent) was observed after 1 h of incubation with sildenafil (Fig. 6, A and B). Quantitative real time RT-PCR showed that the ratio of iNOS/18 S rRNA and eNOS/18 S rRNA was increased by 3.5- and 1.5-fold, respectively, after 1 h of treatment with sildenafil. The expression of iNOS and eNOS protein was also enhanced in a time-dependent fashion (Fig. 6, D–F). This increase in protein expression reached ~2-fold for iNOS and ~1.5-fold for eNOS by 30 min after treatment with sildenafil.

Effect of Sildenafil on Necrosis and Apoptosis in iNOS- or eNOS-deficient Cardiomyocytes—To further establish the direct cause-and-effect relationship between sildenafil-induced cytoprotection and iNOS/eNOS, we used cardiomyocytes de-
derived from mice deficient in iNOS or eNOS (i.e. iNOS-KO or eNOS-KO). As shown in Fig. 7A, pretreatment of B6.129 wild-type mouse cardiomyocytes (the background strain of iNOS-KO) with 1 μmol/liter sildenafil resulted in a decrease in necrosis following SI-RO, i.e. from 24.5 ± 2.2 to 14.7 ± 1.5% of total counted cells (n = 3; p < 0.05). However, the protective effect of sildenafil was completely blunted in the iNOS-KO cardiomyocytes (Fig. 7A). Sildenafil also reduced necrosis in C57BL/6J wild-type cardiomyocytes (the background strain of eNOS-KO mice), as shown by a decrease in myocyte viability from 34.6 ± 2.9 to 14.7 ± 0.5% of total counted cells after SI-RO (n = 3; p < 0.001; Fig. 7B). The sildenafil induced protection was not abolished in eNOS-KO cardiomyocytes as indicated by reduction of necrosis from 32.2 ± 0.7 to 24.7 ± 1.9% of total
counted cells after SI-RO (n = 3; p < 0.05; Fig. 7B). Nevertheless, this protective effect of sildenafil in the eNOS-KO cardiomyocytes, although statistically significant, was not as remarkable as observed in wild-type cardiomyocytes. Similarly, sildenafil significantly reduced TUNEL-positive nuclei in wild-type and eNOS-KO mice (Fig. 7C). The drug had no effect on inhibition of apoptosis in cardiomyocytes from iNOS-KO mice (Fig. 7D).

Effect of Sildenafil on Bcl-2 expression in iNOS- or eNOS-deficient Cardiomyocytes—As shown in Fig. 7 (E–G), pretreatment of B6,129 and C57BL/6J wild-type mouse cardiomyocytes with 1 μM sildenafil enhanced the expression of Bcl-2 after 40 min of SI and 1 h of RO (n = 3; p < 0.05). The increase of Bcl-2 expression by sildenafil was abolished in myocytes derived from iNOS-KO mice (Fig. 7, E and F). Although pretreatment with sildenafil enhanced the Bcl-2 level after SI-RO compared with untreated myocytes derived from eNOS-KO mice, such an increase was not statistically significant (Fig. 7, E and G).

Expression of PDE5 in Cardiomyocytes—The expression of PDE5 in cardiomyocytes was evaluated with three distinct assays. First, using RT-PCR we detected mRNA of PDE5 in the intact heart as well as isolated cardiomyocytes (Fig. 7A). Second, using Western blots we detected the protein expression of PDE5 in mice cardiomyocytes (Fig. 7B). Two splice variants of

![Figure 2](image-url)

**Fig. 2. Effect of sildenafil on inhibition of apoptosis in cardiomyocytes.** Cells were treated with 10 μM sildenafil for 1 h followed by 40 min of SI and 18 h of RO. Apoptotic nuclei were observed using TUNEL assay. A–C, nonischemic control; D–F, after 40 min of SI and 18 h of RO; G–I, pretreatment with 10 μM sildenafil before 40 min of SI and 18 h of RO. A, D, and G, cell morphology; B, E, and H, total nuclei (4',6-diamidino-2-phenylindole staining); C, F, and I, TUNEL-positive myocyte nuclei (stained in green fluorescent color). A significant number of cardiomyocytes underwent apoptosis (i.e. TUNEL-positive; Fig. 2F), whereas sildenafil pretreatment reduced TUNEL-positive nuclei (Fig. 2I). J, bar diagram showing quantitative data from three independent experiments.
PDE5 band (90–100 kDa) were observed, which is consistent with the previously reported PDE5A in dog cardiomyocytes (23). Furthermore, using immunohistochemistry and laser confocal microscopy, the green fluorescence for PDE5A in normal isolated myocyte was clearly evident, thereby confirming the localization of PDE5A in adult mouse cardiomyocytes (Fig. 7G). The green fluorescence color was not observed in the cardiomyocytes without antibody incubation (Fig. 7C) or incubation with blocking peptide (Fig. 7D), primary antibody PDE5A (Fig. 7E), or Alexa 488-labeled anti-rabbit antibody (Fig. 7F).

DISCUSSION

We previously demonstrated a powerful preconditioning-like cardioprotective effect of sildenafil in the rabbit (13) and mouse hearts (14). In the present study, we have shown for the first time that sildenafil directly protects adult cardiomyocytes against necrosis and apoptosis following ischemia-reoxygenation injury. Sildenafil-induced inhibition of apoptosis was evident by the significant decrease in the TUNEL-positive nuclei and preservation of $\Delta \psi_m$, which is essential for production of ATP and cellular homeostasis. These results suggest that vasodilatation caused by sildenafil or the presence of other cell types may not be a prerequisite for the potent preconditioning effect of this drug, at least in the isolated cardiomyocyte model of SI-RO. Sildenafil also induced early up-regulation of Bcl-2/Bax ratio, which may have played an important role in the antiapoptotic effect of the drug.

Sildenafil is a selective potent inhibitor of PDE5 (a cGMP-specific isoform of PDE), which promotes increase in cGMP in vascular smooth muscle cells by preventing its breakdown with PDE5 (3). We recently demonstrated that sildenafil up-regulated iNOS and eNOS in the intact murine myocardium, and delayed protection induced by this drug was completely abolished with 1400W, a selective inhibitor of iNOS (14). In addition, it is now well appreciated that various stimuli such as brief episodes of ischemia (35), endotoxin-derivatives (36), agonists of G protein-coupled receptors (37, 38), and systemic hypoxia (39) induce delayed preconditioning in an iNOS-dependent manner. The present results suggest that the NOS-dependent mechanism in sildenafil-induced protection plays a crucial role in the inhibition of apoptosis and necrosis in the cardiomyocytes also. This is demonstrated by absence of the protective effect of sildenafil both against necrosis and apoptosis in the cardiomyocytes derived from iNOS gene knock-out mice (Fig. 7). Although sildenafil-induced protection remained
statistically significant in eNOS knock-out cardiomyocytes, the magnitude of protection was less than the wild-type mice. These results suggest that NO derived from eNOS may have partially contributed to the signaling mechanism leading to cytoprotection by sildenafil.

Our results (Fig. 6, A–D) also showed a significant increase in mRNA and protein expression of iNOS and eNOS (to a lesser extent). The increase in the levels of iNOS and eNOS proteins was evident at the end of 1 h of preincubation with sildenafil, suggesting that NO was available to trigger the preconditioning effect in the cardiomyocytes. Although the role of NO in the immediate cardioprotection with brief episodes of ischemia (ischemic preconditioning) has been debatable, sildenafil clearly induced early protection in the cardiomyocytes through NO. NO is known to activate guanylate cyclase, resulting in enhanced formation of cGMP. Subsequently, cGMP may activate protein kinase G that in turn opens the $K_{ATP}$ channel, resulting in the cardioprotective effects as reported earlier (40).
FIG. 5. Effect of sildenafil on expression of Bax and Bcl-2 in cardiomyocytes: role of NO signaling. A, representative Western blot showing expression of Bax, Bcl-2, and β-actin (housekeeping gene), during 40 min of SI and 1 h or 18 h of RO in the presence or absence of 10 μM sildenafil. B–D, bar diagrams showing averaged expression of Bax, Bcl-2, and Bcl-2 to Bax ratio after SI and 1- or 18-h RO in the presence or absence of 10 μM sildenafil (Sil) pretreatment. Note that the Bcl-2 to Bax ratio was significantly increased after 1 h of sildenafil treatment (Fig. 4D). E, Western blot showing effect of sildenafil (10 μM) on expression of Bcl-2 and Bax in the presence and absence of an inhibitor of NO synthases, L-NAME (100 μM). F, bar diagram depicting the Bcl-2 to Bax ratio. Note that the increase in Bcl-2 or Bax expression as well as Bcl-2 to Bax ratio with sildenafil was completely reversed by L-NAME (Fig. 4E and F).
FIG. 6. Effect of sildenafil on gene transcription and protein expression of iNOS and eNOS in cardiomyocytes. The total RNA was extracted after treatment for 1 h with 10 μM sildenafil and subjected to RT-PCR as described under "Experimental Procedures." A, representative gel showing iNOS, eNOS, and β-actin; B and C, bar diagram showing the quantitative change of iNOS and eNOS mRNA assessed by real time PCR. Note that the mRNA level of iNOS (Fig. 5B) as well as eNOS (to a lesser extent; Fig. 5C) was significantly increased in sildenafil-treated myocytes. D, iNOS and eNOS proteins were measured at the indicated time points following 1-h incubation with 10 μM sildenafil, using Western analysis. E and F, bar diagrams showing normalized density of iNOS and eNOS expression at each of the time points.
FIG. 7. Effect of sildenafil on necrosis, apoptosis, and Bcl-2 expression in cardiomyocytes derived from iNOS and eNOS gene knock-out mice. Myocytes were prepared from either iNOS gene knock-out mice (iNOS-KO) and their B6,129 wild-type control mice (B6,129 WT), or eNOS gene knock-out (eNOS-KO) mice and their C57BL/6J wild-type control mice (C57BL/6J WT). The myocytes were subjected to 40 min of S1 and 1 h of RO. A and B, cell viability determined by Trypan blue assay. Note that pretreatment with sildenafil (S) significantly decreased the cell necrosis caused by S1-RO in myocytes isolated from B6,129 wild-type mice, whereas this drug failed to protect the cells from iNOS-KO mice (A). On the other hand, sildenafil significantly preserved viability of myocytes from C57BL/6J wild-type mice as well as eNOS-KO mice (B).

C and D, cardiomyocyte apoptosis assessed with TUNEL assay following 40 min of S1 and 18 h of RO. Note that sildenafil significantly reduced TUNEL-positive nuclei in the myocytes from B6,129 wild-type, C57BL/6J wild-type, and eNOS-KO mice (D), whereas the drug provided no inhibition on apoptosis in the myocytes from iNOS-KO mice (C). Sildenafil significantly increased Bcl-2 expression and Bcl-2/Bax ratio following S1-RO in the cardiomyocytes isolated from both strains of wild-type mice (i.e. B6,129 and C57BL/6J (E–G)). In contrast, such an increase in Bcl-2 expression and Bcl-2/Bax ratio was not found in the myocytes from iNOS-KO mice (E and F) and also was attenuated in the eNOS-KO myocytes (E and G).
preliminary results suggest definite involvement of protein kinase G in mediating the anti-necrotic and anti-apoptotic effects of sildenafil in isolated mouse cardiomyocytes.\textsuperscript{2}

One of the salient findings in this study is that sildenafil significantly inhibited apoptosis in the cardiomyocytes (Figs. 2 and 7). It was recently documented that sildenafil could induce apoptosis in B-chronic lymphocytic leukemia cells (41). However, this pro-apoptotic effect of sildenafil appeared to be selective for the leukemic B cells because it did not induce apoptosis in normal B lymphocytes even at a drug concentration as high as 100 $\mu$g/ml (41). It is notable that the protective doses of sildenafil used in the present study are 1 and 10 $\mu$m ($\approx 0.69$ and 6.9 $\mu$g/ml, respectively), which are much lower than used to demonstrate the pro-apoptotic effect in the cancer cells (41). Apoptotic cell death has been appreciated as an important component of the pathogenesis of myocardial ischemia-reperfusion injury (42–46). Our results on sildenafil-induced anti-apoptotic effect in cardiomyocytes may have potential therapeutic ramifications in chronic ischemic heart diseases and heart failure, where apoptosis serves as the primary driving force for the loss of cardiomyocytes. Furthermore, the significant increase in the Bcl-2 to Bax ratio (Fig. 5) is likely to be one of the responsible factors for the inhibitory effect of sildenafil on apoptosis in the cardiomyocytes, because Bcl-2 has been recognized as a key anti-apoptotic protein (47). Interestingly, our results show that either coinubcation with NOS inhibitor, L-NAME (Fig. 5) or genetic deletion of iNOS (Fig. 7) completely abolished the sildenafil-induced Bcl-2 expression in cardiomyocytes. These results established a key link between NO signaling and the expression of cytoprotective antiapoptotic protein, Bcl-2. Studies in neuronal cells also suggested a role for NO in induction and regulation of Bcl-2 (48, 49). In addition, the mitochondrial ATP-sensitive potassium channel opening property of sildenafil (13) may also contribute to its role in the inhibition of apoptosis. A previous report by Akao \textit{et al.} (50) has shown that the openers of the K\textsubscript{ATP} channel \textit{i.e.} diazoxide and pinacidil) were able to reduce apoptosis induced by oxidative stress in the neonatal rat cardiomyocytes.

In the present study, we have provided unequivocal proof of the presence of PDE5 in cardiomyocytes with three assays that detected mRNA, protein expression, and localization of PDE5. There has been a dominant view that PDE5 is not present in the myocardium (11), mainly based on the earlier studies by Ito \textit{et al.} (20) and Wallis \textit{et al.} (21), who failed to find enzyme activity and/or immunoreactive bands of PDE5 in human ventricular tissues. However, this concept was challenged by a

\textsuperscript{2} A. Das and R. C. Kukreja, unpublished observations.
recent study where abundant expression of PDE5 was observed in isolated canine ventricular cardiomyocytes with immunohistochemistry (23). In agreement with this observation, our study demonstrated for the first time the presence of PDE5 in murine ventricular cardiomyocytes (Fig. 8). The discrepancy may be attributed to a possible species difference between human and other mammalian species like dog and mice. However, in the study by Loughney et al. (22), a strong band was also detected for PDE5 mRNA in human heart sample. The presence of PDE5 in guinea pig cardiomyocytes was also suggested by enhanced accumulation of cGMP level without affecting cAMP (51) with zaprinast, a less potent inhibitor of PDE5 than sildenafil (3). Thus it is possible that sildenafil could also enhance cGMP partially through direct inhibition of PDE5 in the mouse cardiomyocytes.

In summary, for the first time, we have shown a direct protective action of sildenafil in isolated adult cardiomyocytes that is independent of any vascular response or presence of other cell types. Our data, based on the pharmacological inhibition of NOS and cardiomyocytes derived from iNOS/eNOS gene knock-out mice, also suggest an important role of NO signaling in protective effect of sildenafil against apoptosis and necrosis. Because of the presence of PDE5 in mouse ventricular cardiomyocytes, its direct inhibition by sildenafil may also contribute to the cytoprotective effect by maintaining cGMP accumulation. Overall, these results provide an important mechanistic basis of sildenafil-induced cardioprotection, which may further give support in exploiting the new therapeutic application of sildenafil and other clinically approved PDE5 inhibitors for treatment of ischemic heart diseases (11, 17).

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