Sildenafil-mediated acute cardioprotection is independent of the NO/cGMP pathway

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Sildenafil was originally developed to treat angina pectoris and subsequently became the first Food and Drug Administration-approved drug for erectile dysfunction. Many recent studies (11, 15, 21, 27, 34) have again focused on the protective effects of sildenafil in animal models of myocardial ischemia-reperfusion (MI/R) injury. Previous studies have suggested that the protective effects of sildenafil are mediated via activation of endothelial nitric oxide (NO) synthesis (eNOS) and inducible NOS (iNOS). To further investigate the protective mechanism of sildenafil, we subjected wild-type, eNOS, and iNOS null animals to 30 min of myocardial ischemia and 24 h of reperfusion. Treatment with 0.06 mg/kg sildenafil 5 min before reperfusion significantly reduced myocardial infarct size in wild-type, eNOS, and iNOS null mice (eNOS+/− and iNOS−/−) animals. Additionally, the low dose utilized in this study did not alter myocardial cGMP. These results suggest that acute low-dose sildenafil-mediated cardioprotection is independent of eNOS, iNOS, and cGMP. As a second series of experiments, we investigated sildenafil in db/db diabetic mice subjected to MI/R. We found that sildenafil failed to protect diabetic mice against MI/R. However, NO− donor therapy was found to significantly protect against MI/R injury in both nondiabetic and diabetic mice, suggesting that protection could be conferred in diabetic mice and that the upstream modulator of soluble guanylyl cyclase, NO−, may mediate protection independent of cGMP signaling. The present study suggests that further research is needed to delineate the precise mechanisms by which sildenafil exerts cardioprotection.

Sildenafil was originally developed to treat angina pectoris and subsequently became the first Food and Drug Administration-approved drug for erectile dysfunction. Many recent studies (11, 15, 21, 27, 34) have again focused on the protective role of sildenafil in numerous cardiac pathologies. Sildenafil is a potent and selective inhibitor of cGMP-specific phosphodiesterase type 5A (PDE-5). PDE-5 is responsible for the degradation of cGMP by hydrolysis to guanosine 5′-monophosphate (5′-GMP). Thereby, inhibition of PDE-5 by sildenafil preserves the cGMP pool and potentiates downstream signaling.

PDE-5 inhibition is currently a major focus of study in myocardial ischemia-reperfusion (MI/R) injury. There have been several proposed mechanisms by which sildenafil may protect against MI/R injury. Most current research has focused on mechanisms of ischemic preconditioning (20, 21) and implicated endothelial nitric oxide (NO) synthase (eNOS) and inducible NOS (iNOS) as prominent players in sildenafil-mediated protection. Sildenafil administered before ischemia has been found to reduce infarct size in an in vivo rabbit model (24). The same group (30) reported that sildenafil upregulated both eNOS and iNOS and that inhibition of iNOS completely abolished the protective effect of sildenafil. Additionally, in a study (9) of adult mouse cardiomyocytes, it was found that sildenafil protected against necrosis and apoptosis in an iNOS- and eNOS-dependent fashion.

Studies utilizing heart failure models have also cited NO synthases as central to the protective effects of sildenafil. Pretreatment with sildenafil attenuated apoptosis and left ventricle (LV) dysfunction in a model of doxorubicin-induced cardiomyopathy (11). Protection was lost with administration of either Nω-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, or 5-hydroxydecanoic acid, a mitochondrial ATP-sensitive K+ (mitoKATP) channel blocker. In an iso-proterenol-induced hypertrophy rat model, it was found that sildenafil reduced myocardial hypertrophy and necrosis. It was found that sildenafil increased cGMP levels and that protection was abrogated with the addition of the NOS inhibitor Nω-nitro-L-arginine (L-NNA) (15).

These findings have been bolstered by reports (6) that sildenafil has no effect on erectile function in eNOS null (eNOS−/−) and eNOS/neuronal NOS (nNOS−/−) mice. In accordance, Takimoto et al. (33) found that PDE-5A inhibition had no effect on β-stimulated contractility in eNOS−/− mice or mice pharmacologically treated with the NOS inhibitor L-NAME or the soluble guanylyl cyclase inhibitor ODQ.

Given the multitude of studies suggesting that protection by sildenafil is eNOS and iNOS dependent, the first aim of the current investigation was to test this hypothesis utilizing eNOS and iNOS null mice in an in vivo model of MI/R. Additionally, all of the studies to date have utilized healthy animals with no preexisting risk factors for cardiovascular disease. We therefore chose to investigate sildenafil in a more clinically relevant model utilizing the db/db diabetic mouse.

MATERIALS AND METHODS

Chemicals and Reagents

Sildenafil citrate [1-((3-[6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo(4,3-d)pyrimidin-5-yl]-4-ethoxyphenyl)sulfonyl)-4-methyl piperazine citrate], a selective inhibitor of cGMP-specific phosphodiesterase type 5 (PDE-5) was administered at doses ranging from 0.03
to 0.25 mg/kg via injection into the LV lumen 5 min before reperfusion dissolved in normal saline.

Dipropylenetriamine (DPTA) NONOate (DPTA/NO), a NO donor, was purchased from Alexis Biochemicals (San Diego, CA) and administered (100 μg/kg) via injection into the LV lumen 5 min before reperfusion dissolved in normal saline.

Animals

Diabetic mice. Our studies focused on the db/db diabetic mouse as an animal model of Type 2 diabetes mellitus. Male B6.Cg-m+/-+Leprdb/J (C57BL6/J background) mice were purchased from Jackson Laboratories and maintained on a normal rodent chow diet. Male mice were used at 8–10 wk of age.

iNOS knockout mice. These mice were purchased from Jackson Laboratories and maintained on a normal rodent chow diet. Male mice on a C57BL6/J background were used at 8–10 wk of age.

eNOS knockout mice. These mice were originally donated by Dr. P. Huang (Massachusetts General Hospital, Boston, MA) and generated in our breeding colony at Albert Einstein College of Medicine (Bronx, NY) on a C57BL6/J background. Male eNOS−/− mice were utilized at 8–10 wk of age. All experimental procedures were approved/reviewed by the Albert Einstein College of Medicine Animal Care and Use Committee.

M/I/R Protocol

Surgical procedures used in the M/I/R protocol (Fig. 1) were similar to methods previously described (17, 18). Briefly, mice were weighed and fully anesthetized via intraperitoneal injections of pentobarbital sodium (50 mg/kg) and ketamine (60 mg/kg). Mice were then orally intubated and connected to a Harvard Apparatus Rodent Ventilator (model 835). Oxygen (100%) was supplied, and body temperature was monitored using a rectal probe thermometer and controlled with an infrared heat lamp. A median sternotomy was performed, and the left coronary artery (LCA) was visualized and ligated proximally to minimize coronary injury induced by occlusion and facilitate reperfusion. The LCA was completely occluded for 30 min, and reperfusion was initiated by removal of the 7-0 suture. Following reperfusion, the chest wound was reapproximated, and mice were extubated and allowed to recover with supplemental oxygen until mobile. All mice received butorphanol tartrate (0.2 mg/kg im) to minimize pain.

Myocardial Infarct Size Determination

After 24 h of reperfusion, the mice were anesthetized as described previously, intubated, and connected to a rodent ventilator. A catheter (PE-10 tubing) was placed in the common carotid artery to allow for Evans blue dye injection. A median sternotomy was performed, and the LCA was religated in the same location as before. Evans blue dye (1 ml of a 3% solution) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the nonischemic zone. The heart was rapidly excised and cross-sectioned into 1-mm-thick sections, which were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride for 5 min at 37°C to demarcate the viable and nonviable myocardium within the risk zone. Each of the five 1-mm-thick myocardial slices was weighed, and the areas of infarction, risk, and nonischemic LV were assessed by a blinded observer using computer-assisted planimetry (NIH Image 1.57). All of the procedures for area at risk (AAR) and infarct size determination have been previously described (17, 18).
ischemia and 24 h of reperfusion (Fig. 4).

**Fig. 4.** Sildenafil-mediated cardioprotection is endothelial nitric oxide (NO) synthase (eNOS)-and inducible NOS (iNOS) independent. ENOS null mice (eNOS<sup>−/−</sup>) and iNOS null mice (iNOS<sup>−/−</sup>) were injected with 0.06 mg/kg sildenafil 5 min before reperfusion. Sildenafil yielded significant protection in eNOS<sup>−/−</sup> (A) and iNOS<sup>−/−</sup> (B) animals. Numbers inside circles are number of animals per group. *P < 0.05 vs. vehicle; **P < 0.01 vs. vehicle; ***P < 0.001 vs. vehicle.

Plasma Sildenafil Concentration

HPLC analysis of plasma samples was carried out by SFBC Analytical Laboratories (North Wales, PA).

**Myocardial cGMP Analysis**

Animals injected with 0.06 mg/kg sildenafil or the vehicle normal saline into the LV lumen were euthanized 30 min after injection, and hearts were removed. The LV was dissected and immediately snap frozen in liquid nitrogen. Snap-frozen samples were analyzed by Cayman Chemical (Ann Arbor, MI) EIA testing service for cGMP (7).

**Statistical Analysis**

Data were analyzed where appropriate by Student’s t-test, one-way ANOVA, or two-way ANOVA with post hoc Tukey and Bonferroni analysis using JMP IN statistical software (SAS Institute). Data are reported as means ± SE. P values <0.05 were considered significant.

**RESULTS**

**Dose Response of PDE-5 Inhibition in MI/R**

To determine the lowest efficacious dose of sildenafil and minimize both nonspecific and hemodynamic effects, a dose-response study was performed (Fig. 2). Sildenafil or vehicle (normal saline) was injected into the LV 5 min before reperfusion at incremental doses ranging from 0.03 to 0.25 mg/kg. Mice receiving vehicle injection displayed a 48.1 ± 3.3% infarct per area at risk (Inf/AAR) following 30 min of ischemia and 24 h of reperfusion. Inf/AAR was significantly (P < 0.05) reduced to 29.7 ± 4.8% in mice receiving 0.25 mg/kg sildenafil vs. vehicle. In mice receiving 0.125 mg/kg sildenafil, Inf/AAR was not reduced (35.3 ± 4.2%). Mice receiving either 0.06 mg/kg or 0.03 mg/kg sildenafil both displayed a very significant reduction in Inf/AAR compared with vehicle (26.3 ± 3.1% and 23.5 ± 3.2%; P < 0.001). The percent AAR per LV (AAR/LV) was similar among all study groups, indicating similar severities of myocardial ischemia.

**PDE-5 Inhibition Reduces MI/R Injury in Wild-Type Mice**

Wild-type mice receiving 0.06 mg/kg of sildenafil citrate displayed a very significant (P < 0.001) reduction in percent Inf/AAR vs. vehicle (49.5 ± 2.6% vs. 26.3 ± 3.1%; Fig. 3). Likewise, infarct per LV (Inf/LV) was also reduced (30.6 ± 1.9% vs. 15.0 ± 1.9%; P < 0.001 vs. vehicle). There was no significant difference in AAR/LV between groups.

**The Protective Mechanism of Sildenafil is Independent of eNOS**

eNOS<sup>−/−</sup> mice were subjected to 30 min of myocardial ischemia and 24 h of reperfusion (Fig. 4A). Mice received either 0.06 mg/kg sildenafil or vehicle 5 min before reperfusion. Sildenafil reduced Inf/AAR in eNOS<sup>−/−</sup> mice by 69.2% (59.1 ± 4.3% vs. 18.2 ± 6.3%; P < 0.001). Inf/LV was also reduced (from 36.5 ± 3.7% to 12.7 ± 3.8%; P < 0.01). AAR/LV was similar for both groups.

**Sildenafil Protects Against MI/R Injury in an iNOS-Independent Fashion**

iNOS<sup>−/−</sup> mice were subjected to 30 min of myocardial ischemia and 24 h of reperfusion (Fig. 4B). Mice received either 0.06 mg/kg sildenafil (n = 9) or vehicle (n = 7) 5 min before reperfusion. Sildenafil reduced Inf/AAR in iNOS<sup>−/−</sup> mice by 50.0% (50.6 ± 7.0% vs. 25.3 ± 5.4%; P < 0.05). Inf/LV was also reduced (from 28.4 ± 4.4% to 14.8 ± 3.1%; P < 0.05). AAR/LV was similar for both groups.

**Sildenafil Does Not Protect Diabetic Mice From MI/R Injury**

In a subsequent study of MI/R injury, diabetic (db/db) mice were given either vehicle (n = 8) or 0.06 mg/kg sildenafil (n = 12) 5 min before reperfusion (Fig. 5). Sildenafil did not significantly reduce Inf/AAR in db/db mice [64.4 ± 3.5% vs. 57.4 ± 3.8; P = not significant (NS)] or Inf/LV (41.3 ± 3.9% vs. 31.5 ± 3.0%; P = NS).

**Sildenafil Did Not Alter Myocardial cGMP Levels in Nondiabetic or Diabetic Mice**

Mice were injected with 0.06 mg/kg sildenafil or normal saline into the LV cavity, and plasma and heart samples were collected 30 min later. Intravascular injection of 0.06 mg/kg sildenafil resulted in a plasma concentration of 1.614 ± 0.376 ng/ml.
ng/ml (P < 0.001 vs. vehicle; Fig. 6A). This low dose, although efficacious, did not significantly alter myocardial cGMP levels in wild-type or diabetic mice (n = 4, Fig. 6B). Nondiabetic mice receiving vehicle displayed cGMP levels of 60.19 ± 3.99 pmol/mg protein compared with 57.52 ± 3.76 pmol/mg in mice receiving sildenafil (P = NS). Likewise, db/db diabetic mice receiving vehicle exhibited cGMP levels of 60.00 ± 4.66 pmol/mg, and sildenafil-treated db/db mice exhibited cGMP levels of 67.08 ± 2.29 pmol/mg of protein (P = NS).

The NO Donor DPTA/NO Protects Against MI/R in Nondiabetic and Diabetic Mice

The well-characterized upstream modulator of cGMP, NO, was evaluated in both nondiabetic and diabetic mice to investigate whether direct NO therapy could be cardioprotective. DPTA/NO (100 μg/kg) was injected into the LV 5 min before myocardial reperfusion in nondiabetic and diabetic mice. Nondiabetic mice (Fig. 7A) displayed a 45.4% reduction in Inf/AAR (44.7 ± 3.0% vs. 24.4 ± 2.7%). In correlation, Inf/LV was also significantly reduced (P < 0.01). Diabetic (db/db) mice receiving DPTA/NO (Fig. 7B) before reperfusion showed a significant decrease in Inf/AAR from 62.5 ± 2.0% to 41.4 ± 3.8% (P < 0.001, vs. vehicle). Inf/LV was also significantly reduced by 38.1% in animals receiving DPTA/NO before reperfusion.

DISCUSSION

Recent studies have reported the protective effects of phosphodiesterase type 5 inhibition in various models of cardiac pathology. Suggested mechanisms responsible for the protective action of sildenafil remain to be fully elucidated. In the present study, we sought to examine some of the proposed cardioprotective mechanisms in an in vivo model of MI/R injury. In addition, we hypothesized that sildenafil may augment cGMP signaling and mediate cardioprotection in a more rigorous and clinically relevant model of MI/R injury (i.e., diabetes mellitus).

Initially, a dose-response study was performed to evaluate the most efficacious dose of sildenafil. Importantly, in all of our studies, sildenafil was injected intravascularly only 5 min before reperfusion, thereby avoiding any preconditioning effect. We found that low dose (0.06–0.03 mg/kg) sildenafil was most effective, reducing infarct by ~50% compared with vehicle-treated animals. Similar doses have been shown to have no effect on hemodynamics (8) or regional myocardial blood flow (26), allowing for the investigation of protection independent of alterations in myocardial oxygen demand. In agreement, we found in a subset of animals that 0.06 mg/kg had no effect on systemic blood pressure or heart rate (data not shown).

To test whether mechanism of action of sildenafil is NOS dependent, we next subjected eNOS and iNOS null animals to MI/R. Interestingly, 0.06 mg/kg sildenafil significantly protected both eNOS−/− and iNOS−/− animals. In eNOS−/− animals, which we have previously reported (32) displayed exacerbated injury and increased infarct compared with wild-type animals, sildenafil injected just before reperfusion lowered Inf/AAR to levels just as low as in wild-type mice. This finding clearly demonstrates that protective action of sildenafil is not mediated via eNOS. This discovery, although contradictory to other studies, is not surprising. First, other studies (9, 11, 15, 30) examining the role of eNOS and iNOS have utilized pharmacological inhibition. These drugs (i.e., L-NAME or L-NNA) have been reported (22) to have many other nonspecific effects in addition to inhibiting NOS. In contrast to the present study, previous studies (8, 15, 24, 30) investigated sildenafil treatment before cardiac insult. Also, these studies investigated delayed preconditioning, which may have allowed for iNOS and/or eNOS to be upregulated and lead to cardio-
Sildenafil-mediated cardioprotection

In the present study, we focused on the acute cardioprotective effect with delivery just before reperfusion in an in vivo model.

We also examined modulation by sildenafil of myocardial cGMP levels yielding unexpected results. In the present study, a single dose of 0.06 mg/kg sildenafil resulted in a mean circulating plasma level, 30 min following injection, of only 1.6 ng/ml. In human subjects, a single oral dose of 100 mg translates into a peak plasma concentration of ~440 ng/ml (Pfizer), 275 times higher than levels achieved in this study. Furthermore, 1.6 ng/ml correlates to a concentration of ~2.4 nM, slightly lower than sildenafil’s IC50 of 3.9 nM. This low, yet effective, dose did not, however, significantly alter myocardial cGMP levels in either nondiabetic or diabetic mice. This suggests that the protective effect of sildenafil at this low dose may be independent of increasing myocardial cGMP levels. In corroboration of these findings, a recent study (34) reported that chronic sildenafil delivery resulting in plasma concentrations ~10 nM reversed cardiac hypertrophy in a murine, transaortic constriction model yet did not alter cGMP levels. Whereas the absolute level of cGMP was not altered by sildenafil, recent reports (3, 5) suggest that the subcellular localization of various cGMP pools (i.e., soluble vs. particulate) may play a prominent role in signal transduction. This leaves open the possibility that sildenafil may modulate the compartmentalization and localization of cGMP within the cardiomyocyte.

To test whether sildenafil would protect animals with pre-existing cardiovascular disease, we utilized the db/db diabetic mouse. Cardiovascular disease is the leading cause of death in diabetes. According to the American Heart Association, people with diabetes mellitus are more likely to develop cardiovascular disease due to multiple risk factors (2, 4). These heightened diabetic risk factors include insulin resistance, obesity, physical inactivity, hypertension, and dyslipidemia. Postmortem studies of diabetic hearts have independently concluded that coronary heart disease is increased three- to fourfold when compared with nondiabetic subjects (13, 29). It has also been found that there is a much higher risk of mortality following myocardial infarction in diabetics compared with nondiabetic patients (12, 23) and that this correlates strongly with an increase in myocardial infarction size (28). Despite the overwhelming evidence that diabetics suffer from larger myocardial infarction and have a greater risk of developing congestive heart failure (31), there have been very few basic science studies designed to examine the mechanisms involved in diabetic myocardial infarction. Our laboratory has previously reported that the extent of myocardial infarction is significantly increased in the db/db mouse (16). We have also recently reported that these animals have exacerbated injury in a model of heart failure with increased mortality and decreased cardiac function (14).

We found the potent protective effect of sildenafil was lost in this clinically relevant model. Diabetic mice receiving sildenafil just before reperfusion displayed no reduction in infarct compared with mice receiving vehicle. To further investigate whether protection could be conferred in the db/db diabetic mouse, we next analyzed the upstream signaling molecule NO. Current dogma (1, 10, 19, 21, 25) suggests that the NO/cGMP pathway is prominent in NO–mediated protection against MI/R injury. Delivery of the NO– donor DPTA 5 min before reperfusion yielded significant protection in both nondiabetic and diabetic mice. This clearly demonstrates that the db/db diabetic mouse can be pharmacologically protected against I/R injury and thus is a viable model to investigate cardioprotection. These results suggest that, whereas sildenafil has been shown to be protective in healthy animals, the translation of this therapy to the clinical setting, where patients present with preexisting conditions, may not prove beneficial. Clearly, additional translational studies are needed to evaluate the efficacy of PDE-5 inhibition in myocardial infarction.

In conclusion, we have shown that low-dose sildenafil significantly reduces MI/R injury in an in vivo murine model. This protection was determined to be independent of both eNOS and iNOS through the use of null animals. Additionally, the dose used in the current study did not alter myocardial cGMP levels. The efficacy of sildenafil was not observed in diabetic mice subjected to MI/R injury. These results suggest that further research is needed to delineate the mechanisms involved in sildenafil-mediated cardioprotection allowing for translation to the clinical setting.

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