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Phosphodiesterase-5 Inhibitor, Tadalafil, Protects Against Myocardial Ischemia/Reperfusion Through Protein-Kinase G–Dependent Generation of Hydrogen Sulfide

Fadi N. Salloum, PhD; Vinh Q. Chau, BS; Nicholas N. Hoke, BS; Antonio Abbate, MD; Amit Varma, MD; Ramzi A. Ockaili, PhD; Stefano Toldo, MS; Rakesh C. Kukreja, PhD

Background—Tadalafil is a novel long-acting inhibitor of phosphodiesterase-5. Because cGMP-dependent protein kinase (PKG) signaling plays a key role in cardioprotection, we hypothesized that PKG activation with tadalafil would limit myocardial ischemia/reperfusion (I/R) injury and dysfunction. Additionally, we contemplated that cardioprotection with tadalafil is mediated by hydrogen sulfide (H_2S) signaling in a PKG-dependent fashion.

Methods and Results—After baseline transthoracic echocardiography (TTE), adult ICR mice were injected i.p. with vehicle (10% DMSO) or tadalafil (1 mg/kg) with or without KT5823 (KT, PKG blocker, 1 mg/kg) or dl-propargylglycine (PAG, Cystathionine-γ-lyase [CSE, H_2S-producing enzyme] blocker; 50 mg/kg) 1 hour before coronary artery ligation for 30 minutes and reperfusion for 24 hours, whereas C57BL wild-type and CSE-knockout mice were treated with either vehicle or tadalafil. After reperfusion, TTE was performed and hearts were collected for infarct size (IS) measurement using TTC staining. Survival was increased with tadalafil (95%) compared with control (65%, $P<0.05$). Infarct size was reduced with tadalafil (13.2±1.7%) compared to vehicle (40.6±2.5%; $P<0.05$). KT and PAG abolished tadalafil-induced protection (IS: 39.2±1% and 51.2±2.4%, respectively) similar to genetic deletion of CSE (47.2±5.1%). Moreover, tadalafil preserved fractional shortening (FS: 31±1.5%) compared to control (FS: 22±4.8%, $P<0.05$). Baseline FS was 44±1.7%. KT and PAG abrogated the preservation of LV function with tadalafil by decline in FS to 17±1% and 23±3%, respectively. Compared to vehicle, myocardial H_2S production was significantly increased with tadalafil and was abolished with KT.

Conclusion—PKG activation with tadalafil limits myocardial infarction and preserves LV function through H_2S signaling. (***Circulation*. 2009;120[suppl 1]:S31–S36.)**

Key Words: phosphodiesterase inhibitors ■ ischemia-reperfusion injury ■ PKG ■ CSE ■ H_2S

Phosphodiesterase-5 (PDE-5) inhibitors including sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis) are FDA-approved for treatment of erectile dysfunction. More recently, sildenafil was indicated for management of pulmonary arterial hypertension.[1] There has been a substantial growth in evidence supporting the cardioprotective role of PDE-5 inhibitors against ischemia/reperfusion injury (I/R). Several studies from our laboratory have shown that sildenafil and vardenafil reduce infarct size, apoptosis, and attenuate cardiac dysfunction after I/R or permanent coronary artery ligation.[2,3,4] However, relatively little work has been done in identifying the role of tadalafil in cardioprotection against I/R. Tadalafil is a selective PDE-5 inhibitor whose effects can last up to 36 hours, whereas the durations of action of sildenafil and vardenafil are generally 4 to 8 hours. Tadalafil is also the only PDE-5 inhibitor whose activity is unaffected by food and has a relatively short time to onset of action (16 to 17 minutes). The pharmacokinetic profile of tadalafil shows maximal plasma concentration within 2.0 hours and an elimination half-life of 17.5 hours. In this respect, the use of tadalafil is quite attractive for long-term management of cardiovascular disease. Moreover, tadalafil is a highly selective inhibitor of PDE with >10 000-fold selectivity for PDE-5 over PDE-1 to PDE-4 and approximately 700-fold selectivity for PDE-5 over PDE-6.[5]

Sesti et al have recently shown that tadalafil reduces infarct size in rats after I/R,[6] but its impact on myocardial contractility remains uncertain. Also, there is currently no information on the mechanism of protection by tadalafil, although sildenafil protects the heart through activation of PKC, expression of endothelial and inducible nitric oxide synthases (eNOS/iNOS), protein kinase G (PKG), and opening of mitochondrial K_{ATP} (mitoK_{ATP}) channels in the heart.[2–4,7–9]

Hydrogen sulfide (H_2S) is a gaseous molecule that is produced enzymatically on a continuous basis at micromolar

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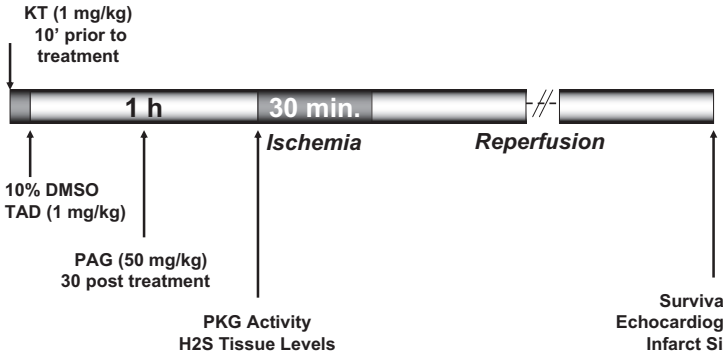

Figure 1. Experimental protocol. Arrows indicate time points for treatment, performance of surgical procedures, and measurement of various parameters.

levels in mammals and exerts a number of physiological actions in the cardiovascular system. Similar to PKG, H_2S has been shown to protect the heart via opening of mitoK_{ATP} channel.[10] The H_2S-producing enzyme, cystathionine-γ-lyase (CSE), is expressed in the heart and administration of the H_2S donor, sodium hydrosulfide, reduces infarct size after I/R. Because PKG and H_2S open mitoK_{ATP} channels, similar to sildenafil and vardenafil,[2,3] we tested the hypothesis that tadalafil might lead to protection against I/R through generation of H_2S from CSE. Interestingly, our results show that tadalafil indeed activates myocardial PKG and also induces cardioprotection against I/R, which was abolished with the PKG inhibitor KT5823 (KT). Moreover, the protective effect of tadalafil was blunted by treatment with a CSE inhibitor, dl-propargylglycine (PAG), as well as in CSE-knockout mice suggesting a definite role of endogenous H_2S signaling in cardioprotection with tadalafil.

Materials and Methods

Animals

Adult male outbred ICR mice were supplied by Harlan Sprague Dawley (Indianapolis, Ind). The mean body weight was 32.2±0.4 g. C57BL mice were supplied by the Jackson Laboratories (Bar Harbor, Me). CSE-knockout mice (4 males and 5 females) were kindly provided by Dr David J. Lefer (Emory University School of Medicine, Atlanta, Ga). All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by National Institutes of Health (No. 85-23, revised 1996).

Drugs and Chemicals

KT5823, dl-propargylglycine, triphenyltetrazolium chloride (TTC), and Dimethyl sulfoxide (DMSO; vehicle for tadalafil) were purchased from Sigma-Aldrich. Tadalafil powder was kindly provided by Lilly ICOS (Indianapolis, Ind).

Myocardial Infarction Protocol

The methodology of myocardial infarction was described previously.[4] In brief, the left descending coronary artery was identified and occluded for 30 minutes by a 7.0 silk ligature that was placed around it and a small piece of polyethylene tubing (PE10) that was positioned on top of it. After coronary artery occlusion for 30 minutes, reperfusion was established by removing the PE10 tube that was compressing the coronary artery. After reperfusion, the air was expelled from the chest and the animals were extubated.

Experimental Groups

Ten groups were used. (1) Dimethyl sulfoxide (DMSO, vehicle for Tadalafil): Each mouse received 0.2 mL 10% DMSO; i.p., 1 hour before I/R; (2) Tadalafil: Mice received an i.p. injection of 1 mg/kg (in 0.2 mL 10% DMSO), 1 hour before I/R; (3) PKG Inhibitor; KT5823 (1 mg/kg; i.p.) + Tadalafil: KT was given 10 minutes before tadalafil, which was administered as in group 2; (4) Tadalafil + CSE Inhibitor PAG (50 mg/kg): PAG was given 30 minutes after tadalafil treatment as in group 2; (5) KT + DMSO control: KT given 10 minutes before DMSO as in group 1; (6) DMSO + PAG control: PAG was given 30 minutes after DMSO administration as in group 1; (7) Sham: Mice were subjected to a left thoracotomy without coronary artery ligation as a control for the surgical procedure (the animals in this group received no treatment until sampling of the heart); (8) DMSO-treated C57BL wild-type mice (control for CSE-knockout): Each mouse received DMSO as in group 1; (9) Tadalafil-treated C57BL wild-type mice: Mice received tadalafil as in group 2; (10) Tadalafil in CSE-knockout mice: Six mice (4 male and 2 female) received tadalafil as in group 2. In Groups 1 to 6 and 8 to 10, infarct size was measured 24 hours after I/R. Before sacrifice, LV function was analyzed using echocardiography. Six to 8 mice in each group were used for infarct size assessment and for functional analysis using echocardiography. The detailed experimental protocol is shown in Figure 1. Three additional mice in groups 1 and 2 were used for measurement of hemodynamics throughout preischemia, ischemia and reperfusion.

Survival

Survival rate was determined based on the animals that survived the experimental protocol starting at recovery afater surgery until 24 hours after infarction.

Infarct Size Assessment

After 24 hours of reperfusion, the heart was quickly removed and mounted on a Langendorff apparatus as described previously.[4] The areas of infarcted tissue, risk zone, and whole left ventricle were determined using TTC staining by computer morphometry using a Bioquant imaging software.

Hemodynamics

Systemic hemodynamics including systolic, diastolic, and mean blood pressures as well as heart rate were measured using a noninvasive tail cuff volume pressure recording system (CODA-2, Kent Scientific) in the anesthetized mouse. Tail cuff inflation was performed on 20 occasions before measurements were taken. The mean of a minimum of 30 recordings on each occasion was taken, and the mean data were compared between the groups.

PKG Activity

Mice were divided into 3 groups: (1) No treatment; (2) 2% to 10% DMSO; and (3) Tadalafil 1 hour before heart explantation. PKG activity was measured according to the manufacturer's instructions (Cyclex). Spectrophotometric absorbance was measured at 450 nm, and the results were normalized per mg of protein.

Cardiac Tissue Levels of H_2S

A subset of 3 mice per group was used for H_2S measurement. Group 1 was treated with 10% DMSO as control; Group 2 was treated with tadalafil 1 hour before heart collection; and Group 3 was given KT, 10 minutes before tadalafil as in group 2. The tissue concentration of H_2S was measured by homogenizing snap-frozen hearts in 1 mL of

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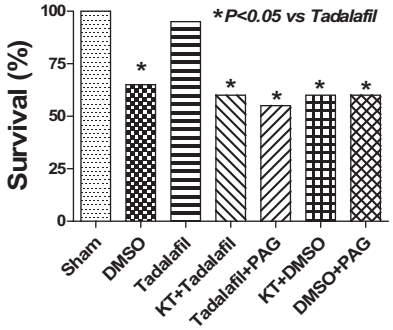

Figure 2. Survival of mice after I/R in the various treatment groups at 24 hours post-MI. Note that tadalafil-treated mice exhibited a significant increase in survival compared with animals treated with vehicle or blocker of PKG and CSE ($P<0.05$).

100 mmol/L potassium phosphate buffer (pH 7.4). To trap H_2S, 250 μL of zinc acetate (1% wt/vol) was added to the tissue homogenate followed by 30 minutes incubation at 37°C. The reaction was stopped by adding 250 μL of trichloroacetic acid (10% wt/vol) to the assay mixture and incubated for 60 minutes at 37°C before centrifugation at 14 000 *g* for 10 minutes. H_2S concentration of the supernatants was measured using a highly specific H_2S sensor connected to a single channel analyzer (Apollo 1000, WPI) and was calculated using a calibration curve of NaHS standards. Protein concentration was measured spectrophotometrically at 595 nm. The results are expressed as μM/mg of protein.[11]

Echocardiography

Echocardiography was performed using the Vevo770 imaging system (VisualSonics, Inc) before surgery (baseline) and 24 hours after surgery before sacrificing the animal. Pentobarbital (30 mg/kg; i.p.) was used for anesthesia, and the procedure was carried out as previously described[4] to measure LV end-diastolic diameter (LVEDD) and end-systolic diameter (LVESD). LV fractional shortening (FS) was calculated as (LVEDD−LVESD)/LVEDD×100.

Statistics

All measurements of infarct size and risk areas are expressed as group means±SE. Changes in echocardiography parameters and infarct size were analyzed using the random effects ANOVA for repeated-measures to determine the main effect of time, group, time-by-group interaction, and the post-hoc 2-sided Dunnett test to compare 2 groups at a time. Statistical differences were considered significant if the probability value was <0.05. Discrete variables were presented as absolute and percent value. The χ^2 test (or the Fisher exact test when appropriate) was used to compare discrete variable in different groups. The Bonferroni correction for posthoc analysis was used when comparing 2 groups from 3 or more groups.

Statement of Responsibility

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Survival

A total of 135 mice were used in this study. Nineteen of 20 mice survived with tadalafil (95%) as compared to 13 of 20 with DMSO (65%, $P<0.05$, Figure 2). PKG and CSE inhibition with KT and PAG in addition to tadalafil reduced survival rate to 60% and 55%, respectively, $P<0.05$ versus tadalafil. KT and PAG alone had no adverse effects on survival as compared with DMSO ($P>0.05$). The survival rate was 100% in sham-operated mice. Six of 7 C57BL mice (the controls for CSE-knockout mice) survived in the tadalafil group (86%), and 6 of 10 survived in the DMSO group (60%). All 4 males and only 2 females survived of a total of 9 CSE-knockout mice treated with tadalafil (67%).

Infarct Size

Infarct size (% of risk area) was reduced from 40.6±2.5 with DMSO to 13.2±1.7 with tadalafil 24 hours after infarction ($P<0.05$, Figure 3A). The infarct-sparing effect of tadalafil

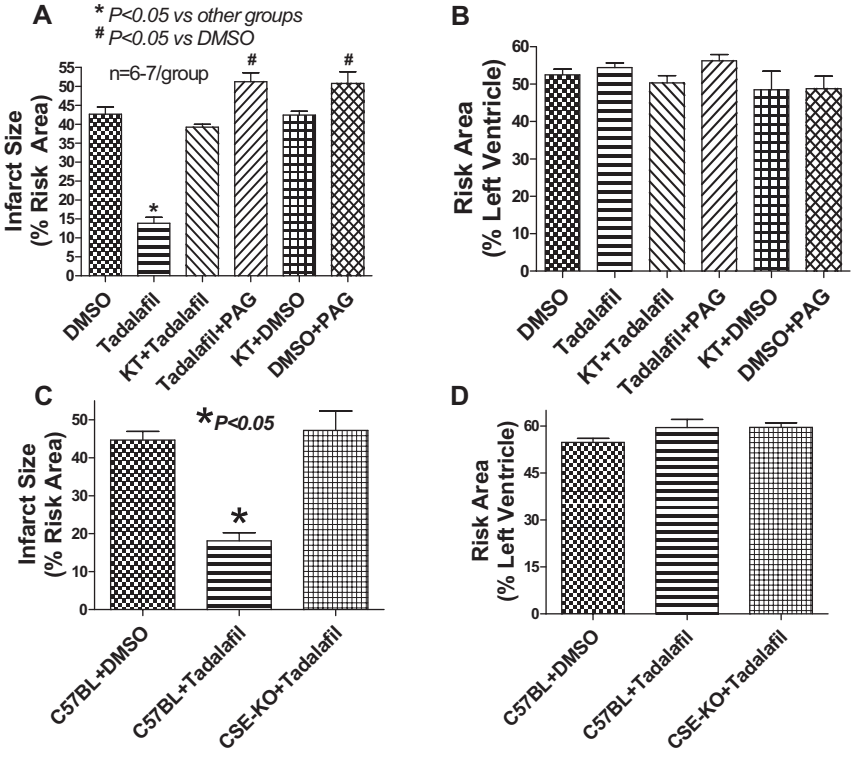

Figure 3. A, Myocardial infarct size (% of RA) measured 24 hours post-MI in the various groups. Note that infarct size was significantly reduced with tadalafil, which was blocked by KT and PAG. B, The area-at-risk, expressed as percent of the left ventricle, was similar in all groups. C, Myocardial infarct size measured 24 hours post-MI in C57BL wild-type mice, mice treated with DMSO or tadalafil, and in CSE-knockout mice. Note that genetic deletion of CSE abrogated the infarct-sparing effect of tadalafil. D, The area-at-risk was similar in C57BL wild-type mice, mice treated with DMSO or tadalafil, and in CSE-knockout mice.

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Table. Hemodynamics

	DMSO			Tadalafil		
Group	Baseline	Ischemia	Reperfusion	Baseline	Ischemia	Reperfusion
SBP	98±6	78±3*	93±5	94±4	81±4	94±3
DBP	70±4	56±6	72±4	71±3	58±4	73±3
MAP	79±3	63±5*	79±2†	77±2‡	65±3	80±2‡
HR	524±21	496±38	502±16	517±41	526±42	558±28

Values are means±SEM. SBP indicates systolic blood pressure (mm Hg); DBP, diastolic blood pressure (mm Hg); MAP, mean arterial blood pressure (mm Hg); HR, heart rate (bpm).

*$P<0.05$ versus Baseline; †$P<0.05$ versus Ischemia DMSO; ‡$P<0.05$ versus Ischemia Tadalafil.

was abolished with KT as shown by an increase in infarct size to 39.2±1.0 ($P<0.05$). Animals treated with KT alone had an infarct size of 40.9±0.9, which was not different from the DMSO group ($P>0.05$). PAG not only blunted the cardioprotection exerted by tadalafil, but exacerbated I/R injury as shown by an increase in infarct size to 51.2±2.4 ($P<0.05$ versus DMSO). Control animals treated with PAG alone had a similar increase in infarct size to 51.5±4.7 ($P<0.05$ versus DMSO). The risk areas (% LV) were not statistically different between the groups (Figure 3B). Sham-operated mice did not exhibit any infarction. Genetic deletion of CSE abrogated the infarct-sparing effect of tadalafil (47.2±5.1%) compared to wild-type mice treated with tadalafil (18.1±2.1%, $P<0.05$, Figure 3C).

Hemodynamics

The systemic mean arterial pressure decreased immediately after opening of the chest (data not shown) and during ischemia, but was restored to baseline after reperfusion and closing of the chest. Administration of tadalafil did not cause marked changes in hemodynamic parameters at 1 hour after treatment. Except at the indicated time points, the mean values were not significantly different between the groups (Table).

PKG Activity

Treatment with tadalafil caused increase in cardiac PKG activity (A450/mg protein) to 154.6±25.5 ($P<0.05$) as compared with sham (60.7±6.4) and DMSO-treated mice (83.5±10.8; Figure 4).

Cardiac Tissue Levels of H_2S

Figure 5 illustrates a significant increase in H_2S levels with tadalafil treatment (3.1±0.2 μmol/L/mg, $P<0.05$) as compared to DMSO (0.7±0.3 μmol/L/mg). Treatment with KT before tadalafil abrogated the increase in H_2S seen with tadalafil alone to 0.7±0.1 μmol/L/mg, which is not different from DMSO control ($P>0.05$).

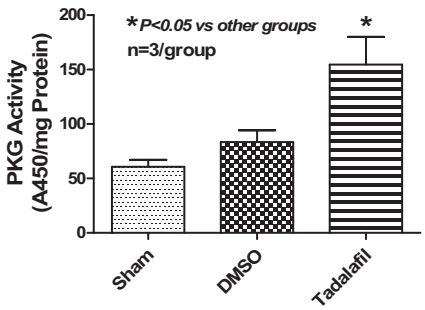

Figure 4. PKG activity 1 hour after treatment with tadalafil or 10% DMSO compared with sham hearts. Note that tadalafil increases PKG activity compared with DMSO ($P<0.05$).

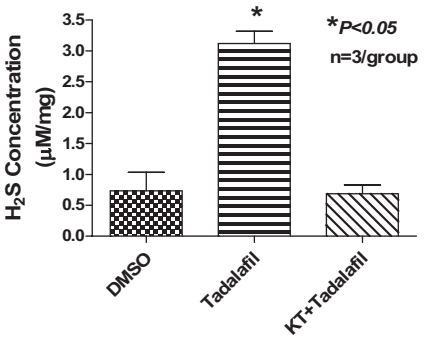

Figure 5. H_2S tissue levels assessed in hearts harvested 1 hour after treatment. Note that tadalafil caused an increase in cardiac H_2S compared with DMSO ($P<0.05$). Interestingly, KT abrogated the increase in H_2S caused by tadalafil ($P<0.05$).

Left Ventricular Remodeling and Function

Figure 6A shows representative M-mode images from sham-operated, DMSO (vehicle)-treated, tadalafil-treated, KT+tadalafil–treated, and tadalafil+PAG–treated mice at 24 hours after MI. None of the groups presented with significant LV dilatation at 24 hours after infarction (Figure 6B), however tadalafil decreased LV end-systolic diameter (LVESD: 2.5±0.1 mm) and preserved fractional shortening (FS: 31±1.5%) as compared to DMSO (LVESD: 3.1±0.2 mm and FS: 22±4.8%, respectively; $P<0.05$). Baseline LVESD and FS were 2.0±0.1 mm and 44±1.7%, respectively, and KT administration before tadalafil abrogated the preservation of LV function as demonstrated by marked increase in LVESD to 3.3±0.1 mm and a decline in FS to 17±1% ($P<0.05$ versus tadalafil). Similarly, PAG treatment blocked the functional improvement in tadalafil-treated mice as shown by an increase in LVESD to 3.0±0.2 mm and a deterioration in FS to 24±3% ($P<0.05$; Figure 6C and 6D).

Discussion

The cytoprotective effects of the gaseous molecules, NO and carbon monoxide (CO), against I/R have been well documented in the literature.[12,13] H_2S is well known as a toxic gas often used to describe the smell of rotten eggs.[14] The production of H_2S in mammalian systems has been attributed to 2 key enzymes, CSE and cystathionine β-synthase (CBS). CSE and CBS are heme-containing enzymes whose activities are dependent on the cofactor pyridoxal 5′-phosphate. CBS is capable of catalyzing the reaction of cysteine with other free thiols to generate H_2S; and likewise thiocysteine generated by CSE can interact with thiols to generate H_2S.[15] CSE is physiologically activated by calcium-calmodulin, a mechanism for H_2S formation in response to vascular activation.[16] It has been shown that genetic deletion of this enzyme in mice markedly reduces H_2S levels in the serum, heart, aorta, and other tissues, and these animals display pronounced hypertension and diminished endothelium-dependent vasorelax-

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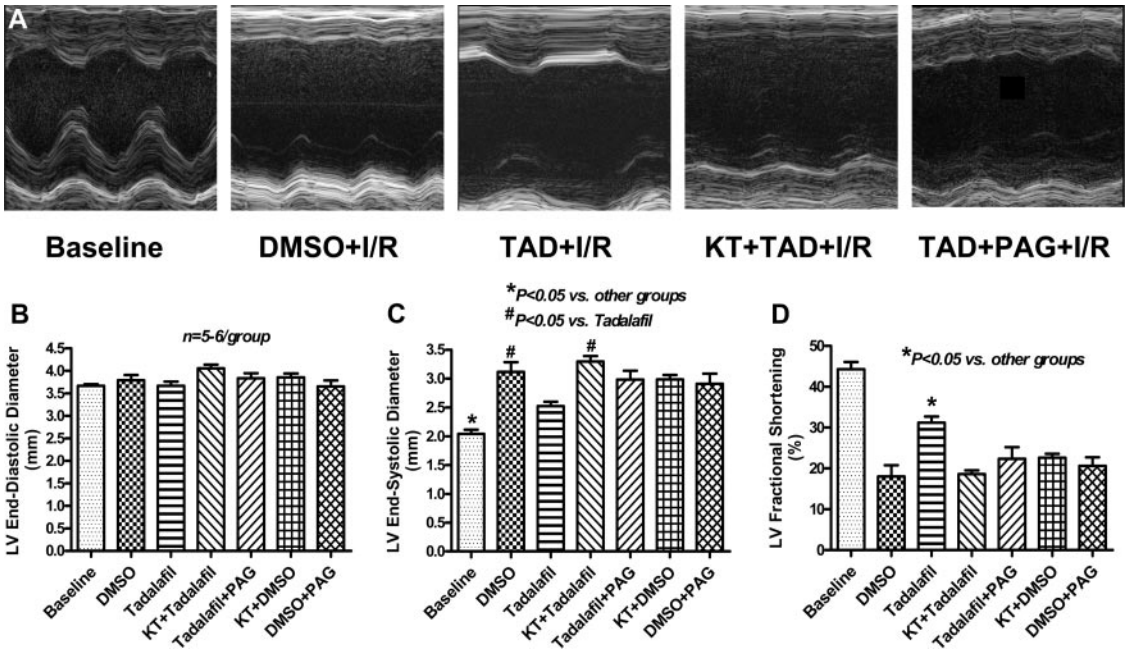

Figure 6. A, Representative M-mode images demonstrating the preservation of LV contractility with tadalafil compared with vehicle. KT and PAG abolished this preservation in LV function. B, LV end-diastolic diameter, end-systolic diameter, and fractional shortening measured in the various treatment groups.

ation. In the present study, we investigated the potential role of H_2S generated from CSE in tadalafil-induced protection against I/R injury. Our results show that tadalafil significantly reduced infarct size and attenuated LV dysfunction at 24 hours after infarction as shown by preservation of fractional shortening and improvement in survival of mice. Moreover, tadalafil-induced cardioprotection was associated with increased activity of PKG and enhanced generation of endogenous H_2S through a PKG-dependent pathway. The infarct-sparing effect of tadalafil was abolished with the PKG inhibitor KT, the CSE inhibitor PAG, as well as in CSE-knockout mice. To our knowledge, this is the first investigation that has (1) implicated H_2S as a mediator of cardioprotection with tadalafil, and (2) uncovered a potentially novel role of PKG in generation of H_2S by tadalafil.

PDE-5 inhibition prevents the breakdown of cGMP into 5′GMP, which leads to cGMP accumulation. In addition to its presence in the cavernosal tissue and penile arteries, PDE-5 also exists in vascular smooth muscle cells and murine ventricular cardiomyocytes.[17] We have shown that PDE-5 inhibition increases cGMP levels and activates PKG, which plays an essential role in sildenafil-induced cardioprotection. We also demonstrated that direct adenoviral overexpression of PKG Iα (the isozyme expressed in the heart) in cardiomyocytes induced both eNOS/iNOS and protected them against simulated I/R,[18] and reduced infarct size after I/R in vivo.[19] The increases in PKG activity and expression by sildenafil were responsible for enhanced Bcl-2/Bax ratio, phosphorylation of Akt, ERK1/2, and inactivation of GSK-3ß that eventually resulted in attenuation of necrosis and apoptosis after simulated ischemia/reoxygenation.[10] The evidence that PKG is involved in tadalafil-induced protection is illustrated by a complete loss of the cardioprotective effect with the PKG inhibitor KT in terms of infarct size reduction, prevention of LV systolic dysfunction, and increased survival rate. Tadalafil caused a significant increase in PKG activity at 1 hour after treatment, which is consistent with the induction of ischemia in mice that underwent I/R.

PKG is a serine/threonine protein kinase and is one of the major intracellular receptors for cGMP. There are 2 types of PKG present in eukaryotic cells: type I and type II. PKG is present in high concentrations in smooth muscle, platelets, cerebellum, hippocampus, dorsal root ganglia, neuromuscular end plate, and the kidney vasculature.[20] Activation of PKG phosphorylates many intracellular proteins and regulates important physiological functions such as relaxation of vascular smooth muscle, inhibition of cell differentiation and proliferation, and inhibition of platelet aggregation and apoptosis.[21] Exactly how PKG activation is associated with increased H_2S generation by tadalafil is not clear from the present study, although it may be related to PKG-dependent enhancement of CSE activity in the heart. There is evidence that specificity protein 1 (SP-1; also known as Sp1 transcription factor) plays an important role in the basal transcriptional activity of CSE enzyme.[22] PKG can phosphorylate Sp1 on serine residue(s) which results in transcriptional activation of Sp1 in human SW480 colon cancer cells[23] with consequent increase in CSE activity and possibly generation of H_2S. It is not known whether PKG-dependent transcriptional activation of Sp1 also occurs in the heart. The validity of this proposed mechanism will be addressed in future studies (Figure 7).

H_2S has been suggested to regulate cardiovascular homeostasis and promote cellular signals that modulate metabolism, cardiac function, and cell survival. In fact, moderate use of H_2S balneotherapy (treatment by bathing) has been shown to raise the tolerance to exercise and reduce the daily need for short-acting nitrates in patients with coronary artery disease (CAD).[24] Clinically, H_2S plasma levels in CAD patients are reported to be

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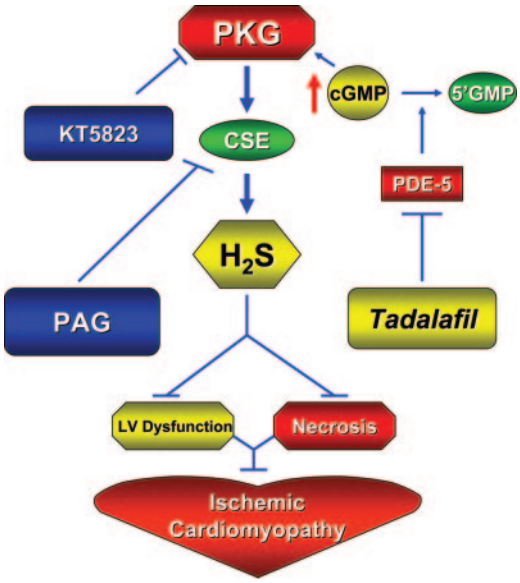

Figure 7. Proposed scheme outlining the pathway by which tadalafil may lead to PKG-dependent generation of H_2S and protection against ischemia/reperfusion injury, LV dysfunction, as well as ischemic cardiomyopathy.

lower than in angiographically normal control subjects.[25] Therefore, the use of pharmacological agents, particularly PDE-5 inhibitors, to augment endogenous H_2S synthesis for combating CAD is quite attractive because of the overall safety margin of this class of drugs. Moreover, the implication of H_2S in tadalafil-induced cardioprotection suggests a potential role of this gaseous molecule in the therapeutic effect of tadalafil for erectile dysfunction as well. In fact, a pilot study suggested that endogenous H_2S plays a role in erectile physiopharmacology.[26] In these studies, the administration of PAG resulted in significant reduction in cavernous nerve stimulation-evoked perfusion pressure.

In conclusion, the present study has provided evidence for a novel mechanism by which the long-acting PDE-5 inhibitor, tadalafil, exerts cardioprotective effects in mice. We believe that other PDE-5 inhibitors including sildenafil and vardenafil might share a similar pathway of PKG-dependent generation for H_2S in protection against I/R injury and possibly post-MI–induced heart failure.

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Disclosures

None.

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Correction

In the article, "Phosphodiesterase-5 Inhibitor, Tadalafil, Protects Against Myocardial Ischemia/Reperfusion Through Protein-Kinase G-Dependent Generation of Hydrogen Sulfide," by Fadi N. Salloum et al, which appeared in the September 15, 2009 Supplement issue of the Journal (*Circulation.* 2009;120:S31–S36), the e-mail address for Dr Salloum in the correspondence footnote should read: fnsalloum@vcu.edu

This change has been made to the current online version of the article.

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