ERK phosphorylation mediates sildenafil-induced myocardial protection against ischemia-reperfusion injury in mice

Anindita Das, Fadi N. Salloum, Lei Xi, Yuan J. Rao, and Rakesh C. Kukreja

Division of Cardiology, Department of Internal Medicine, Virginia Commonwealth University, Richmond, Virginia

Submitted 29 January 2009; accepted in final form 9 March 2009

Das A, Salloum FN, Xi L, Rao YJ, Kukreja RC. ERK phosphorylation mediates sildenafil-induced myocardial protection against ischemia-reperfusion injury in mice. Am J Physiol Heart Circ Physiol 296: H1236–H1243, 2009. First published March 13, 2009; doi:10.1152/ajpheart.00100.2009.—Sildenafil, a selective inhibitor of phosphodiesterase type 5, induces powerful protection against myocardial ischemia-reperfusion injury through activation of cGMP-dependent protein kinase (PKG). We further hypothesized that PKG-dependent activation of survival kinase ERK may play a causative role in sildenafil-induced cardioprotection via induction of endothelial nitric oxide synthase (eNOS)/inducible nitric oxide synthase (iNOS) and Bcl-2. Our results show that acute intracoronary infusion of sildenafil in Langendorff isolated mouse hearts before global ischemia-reperfusion significantly reduced myocardial infarct size (from 29.4 ± 2.4% to 15.9 ± 3.0%; P < 0.05). Cotreatment with ERK inhibitor PD98059 abrogated sildenafil-induced protection (31.8 ± 4.4%). To further evaluate the role of ERK in delayed cardioprotection, mice were treated with sildenafil (ip) 24 h before global ischemia-reperfusion. PD98059 was administered (ip) 30 min before sildenafil treatment. Infarct size was reduced from 27.6 ± 3.3% in controls to 7.1 ± 1.5% in sildenafil-treated mice (P < 0.05). The delayed protective effect of sildenafil was also abolished by PD98059 (22.5 ± 2.3%). Western blots revealed that sildenafil significantly increased phosphorylation of ERK1/2 and GSK-3β and induced iNOS, eNOS, Bcl-2, and PKG activity in the heart 24 h after treatment. PD98059 inhibited the enhanced expression of iNOS, eNOS, and Bcl-2 and the phosphorylation of GSK-3β. PD98059 had no effect on the sildenafil-induced activation of PKG. We conclude that these studies provide first direct evidence that PKG-dependent ERK phosphorylation is indispensable for the induction of eNOS/iNOS and Bcl-2 and the resulting cardioprotection by sildenafil.

myocardial infarction; nitric oxide synthase; phosphodiesterase inhibitor; protein kinases; signal transduction; extracellular signal-regulated kinase

SILDENAFIL CITRATE (Viagra), the first orally active and highly selective inhibitor of cGMP-specific phosphodiesterase type 5 (PDE-5), was approved for treating erectile dysfunction. It exhibited cardioprotective action against ischemia-reperfusion injury in both in situ and isolated hearts (15, 19, 20). In the past few years, our laboratory demonstrated that sildenafil induced acute and delayed cardioprotection against ischemia-reperfusion injury through enhancement of nitric oxide (NO) generation by increased expression of endothelial NO synthase (eNOS)/inducible NO synthase (iNOS), activation of PKC, and opening of mitochondrial ATP-sensitive K⁺ channels (4, 15, 19). Sildenafil also attenuated necrosis as well as apoptosis in cultured adult mouse cardiomyocytes following simulated ischemia-reoxygenation through preserving mitochondrial membrane potential and NO-dependent upregulation of Bcl-2-to-Bax ratio (5). Most recently, we reported that sildenafil-induced protection is dependent upon activation of cGMP-dependent protein kinase (PKG) in adult mouse heart and cardiomyocytes (6). This notion is based on the fact that PKG inhibitors and selective knockdown of PKG by adenoviral vector containing short-hairpin RNA of PKG abolished the antinecrotic and antiapoptotic effect of sildenafil in cardiomyocytes, and PKG inhibition also abrogated the infarct size reduction by sildenafil in isolated mouse hearts. However, the downstream targets of PKG in sildenafil-induced cardioprotection remain incompletely understood.

It has been suggested that ERKs, one of the subfamilies of MAPKs, are the most ideal candidates among the protein kinases that determine the cellular transcriptional activities, cell proliferation, differentiation, and cell survival (12). ERK1/2 cascade has been shown to be involved in ischemic preconditioning (17, 23) and delayed pharmacological preconditioning (10) and plays a critical role in myocardial protection against ischemia-reperfusion injury. Interestingly, our recent study uncovered that acute treatment of sildenafil upregulates ERK1/2 phosphorylation in a PKG-dependent manner in the mouse heart and isolated cardiomyocytes (6). However, it is unknown whether such an enhanced ERK phosphorylation plays a vital role in the above-mentioned signaling cascade of sildenafil-induced cardioprotection. Therefore, the primary goals of the present investigation were to 1) determine whether a selective inhibitor of ERK would abolish the sildenafil-induced early and/or delayed cardioprotection and 2) demonstrate whether ERK inhibition would affect other cardioprotective signaling/effecter components activated by sildenafil, such as eNOS/iNOS, Bcl-2, PKG, and GSK-3β.

MATERIALS AND METHODS

Animals. Adult male outbred ICR mice (average body weight, ~30 g) were supplied by Harlan Sprague Dawley (Indianapolis, IN). The animal care and experiments were conducted under the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health (No. 85-23, Revised 1996), and the rodent experiment protocol was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Global ischemia-reperfusion in Langendorff-perfused mouse heart. The methodology of isolated perfused mouse heart has been described previously in details (19, 27). In brief, the animal was anesthetized

Address for reprint requests and other correspondence: R. C. Kukreja, Eric Lipman Chair of Molecular Cardiology, Division of Cardiology, Box 980281, Virginia Commonwealth University, Medical Center, 1101 East Marshall St., Rm. 7-046, Richmond, VA 23298-0281 (e-mail: rakesh@vcu.edu) or A. Das, Division of Cardiology, Box 980281, Virginia Commonwealth University, Medical Center, 1101 East Marshall St., Rm. 7-040, Richmond, VA 23298-0281 (e-mail: adas2@vcu.edu).
with pentobarbital sodium (100 mg/kg, 33 units heparin ip), the heart was quickly removed from the thorax, and the aorta opening was rapidly cannulated and tied on a 20-gauge blunt needle connected to a Langendorff perfusion system. The heart was retrogradely perfused at a constant pressure of 55 mmHg with modified Krebs-Henseleit (K-H) solution, which contains (in mM) 118 NaCl, 24 NaHCO₃, 2.5 CaCl₂, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 11 glucose, and 0.5 EDTA. The perfusion solution was continuously gassed with 95% O₂-5% CO₂ (pH 7.34–7.49) and warmed by a heating/cooling bath. The heart temperature was maintained at 37°C throughout the experiment. A force-displacement transducer (Model FT03; Grass) was attached to the apex through a No. 5 surgical thread and a rigid metal hook. The resting tension of the isolated heart was adjusted to ~0.30 g. Ventricular contractile force was recorded by a Powerlab 8SP computerized data acquisition system connected to the force transducer. Coronary flow rate was calculated by timed collection of the outflow perfusate. The hearts were not paced.

**Experimental groups and protocol.** The study protocols used to determine the role for ERK in both the early and delayed phase of sildenafil-induced cardioprotection are shown in Fig. 1. In the early phase study, after 20 min of stabilization, the Langendorff-perfused hearts were subjected to 10 min of intracoronary infusion of a recently established cardioprotective dose of sildenafil (pure powder provided by Pfizer; 1 μM at 0.25 ml/min pump speed; see Ref. 6) with or without PD98059 (3 μM; Sigma-Aldrich) or K-H buffer alone followed by 20 min of no-flow global ischemia and 30 min of reperfusion. The ex vivo dose of 1 μM sildenafil is equivalent to ~666 ng/ml, which is comparable with the mean maximal plasma concentration (Cₘₐₓ) of sildenafil reported in clinical studies following the oral dose of 100 mg sildenafil citrate. Particularly, the Cₘₐₓ of 327 ± 236 ng/ml (2) and 514 ng/ml (14) were observed following 100 mg oral dose of sildenafil citrate in clinical studies.

For the delayed phase study, PD98059 (1 mg/kg) or volume-matched DMSO (solvent of PD98059) was administered intraperitoneally 30 min before sildenafil treatment (0.71 mg/kg ip), a well-confirmed dose that induces delayed infarct-limiting cardioprotection in mice; see Refs. 19 and 25). Twenty-four hours after the sildenafil injection, the hearts were isolated and subjected to the same ex vivo global ischemia-reperfusion protocol.

**Measurement of infarct size.** At the end of reperfusion, the heart was immediately removed from the Langendorff apparatus, weighed, and immediately stored at −20°C. The frozen heart was cut from apex to base into 7 or 8 transverse slices (~1 mm thickness), and the slices were incubated in 10% triphenyltetrazolium chloride in phosphate buffer at room temperature for 30 min. The heart slices were then fixed by 10% formaldehyde for ~4 h before areas of infarcted tissue were measured via computer morphometry (Bioquant). The risk area was calculated as total left and right ventricular area minus the area of the cavities. The infarct size was presented as a percentage of risk area. Western blot analysis. Total soluble protein was extracted from whole heart tissue with radioimmunoprecipitation assay buffer (6). The homogenate was centrifuged at 14,000 g for 15 min under 4°C, and the supernatant was recovered. Protein (50 μg) from each sample was separated by SDS-PAGE and transferred onto nitrocellulose.
membrane. The membrane was incubated with primary antibody at a dilution of 1:1,000 for each of the respective proteins, i.e., phosphorylation of ERK (pERK), ERK, iNOS, eNOS, Bcl-2, Bax (rabbit polyclonal), actin (goat polyclonal; Santa Cruz Biotechnology), pGSK-3β (Ser9), GSK-3β, and phospho-vasodilator-stimulated phosphoprotein (pVASP; Ser239) and VASP (rabbit polyclonal; Cell Signaling Technology). The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000 dilutions; 1 h at room temperature). The blots were developed using a chemiluminescent system (ECL Plus; Amersham Biosciences).

**Statistics.** All measurements are expressed as means ± SE. The differences between groups were analyzed with one-way ANOVA.
followed by Student-Newman-Keuls post hoc test for pair-wise comparison. $P < 0.05$ was considered to be statistically significant.

RESULTS

Myocardial infarction. In the early phase study, intracoronary administration of sildenafil (1 μM, 10 min) before the global ischemia-reperfusion reduced infarct size from $29.4 \pm 2.4\%$ in the control group to $15.9 \pm 3.0\%$ ($P < 0.05$; $n = 6$, Fig. 2A). The sildenafil-induced infarct-limiting protection was completely abolished by the ERK inhibitor PD98059 (infarct size, $31.8 \pm 4.4\%$; Fig. 2A). In the delayed phase study, a significant reduction of infarct size with sildenafil ($7.1 \pm 1.5\%$) was also observed compared with the controls ($27.6 \pm 3.3\%$; $P < 0.05$, $n = 6$; Fig. 2B). Again, PD98059 completely...
blocked the delayed infarct-limiting effect of sildenafil (infarct size, 22.18 ± 2.76%). PD98059 alone did not alter the infarct size compared with control in either the early or delayed phase (i.e., 23.5 ± 3.6% and 28.1 ± 2.76%, respectively).

Cardiac hemodynamic and contractile function. There was no significant difference in the preischemia basal functional parameters (i.e., developed force, rate-force product, and resting tension) between the groups in both early and delayed phase studies. Acute treatment of sildenafil tended to improve the posts ischemic rate-force product and coronary flow rate compared with control (Fig. 3, A and C), although the improvement failed to reach the statistical significance mainly because of the higher intragroup variability for the contractile function parameters ($P > 0.05$ with one-way ANOVA). Sildenafil plus PD98059 did not alter the contractile function and posts ischemic coronary flow rate at the early and delayed phases compared with control (Fig. 3). However, PD98059 alone depressed the rate-force product compared with control during the early and delayed phases (Fig. 3, A and B). Posts ischemic coronary flow was not affected by PD98059 alone (Fig. 3, C and D).

Phosphorylation of ERK1/2. Phosphorylation at both threonine 202 and tyrosine 204 residues is required for full enzymatic activation of ERK1/2 (10). With the use of pERK1/2 (Thr202/Tyr204) antibody, Western blot analysis revealed that sildenafil induced the phosphorylation of ERK1/2 in the heart after 24 h of treatment compared with control (Fig. 4A). Treatment with PD98059 blocked sildenafil-mediated phosphorylation of ERK. The densitometric scanning confirmed the significant increase of the ratios of phosphorylation of ERK1 and ERK2 to total ERK after 24 h of sildenafil treatment, which was abolished by PD98059 (Fig. 4, B and C).

Phosphorylation of VASP.PKG activity in the whole heart lysate was measured using the phosphorylation levels of VASP in the heart after 24 h of treatment with sildenafil in the presence or absence of PD98059. Since phosphorylation of VASP at Ser239 is highly selective for PKG, we used a phospho-specific antibody that recognized VASP at the Ser239 position. Our result showed that phosphorylation of VASP significantly increased after 24 h of treatment with sildenafil compared with the control (Fig. 5, A and B). However, treatment with sildenafil in combination with PKG inhibitor...

--

**Fig. 8. Expression of Bcl-2 and Bax.** A: representative Western blot showing the expression of Bcl-2, Bax, and actin in the mouse hearts after 24 h of treatment with sildenafil (Sild) with or without PD98059 (PD). B–D: densitometry analysis of the ratio of Bcl-2 to actin, Bax to actin, and Bcl-2 to Bax, respectively.
PD98059 failed to block the sildenafil-induced phosphorylation of VASP with respect to total VASP (Fig. 5, A and B). In other words, because both the phosphorylated and total VASP were slightly reduced by PD98059, the ratio of pVASP to total VASP in the sildenafil + PD98059 group remains similar to that of the sildenafil group (Fig. 5B). These data and those of a recent study from our laboratory (6) clearly support the notion that sildenafil-induced PKG activation is an upstream event of ERK phosphorylation.

Expression of iNOS and eNOS. Sildenafil enhanced the expression of iNOS and eNOS proteins significantly after 24 h treatment with sildenafil in the intact heart compared with control (P < 0.01, n = 3; Fig. 6). Both iNOS and eNOS induction by sildenafil were inhibited by PD98059 (Fig. 6).

Phosphorylation of GSK-3β. GSK-3β becomes inactivated when phosphorylated at Ser9 (an N-terminal serine residue). The phosphorylation of GSK-3β was significantly increased after 24 h treatment with sildenafil compared with control (P < 0.01, n = 3; Fig. 7, A and B), which was completely inhibited by ERK inhibitor PD98059.

Ratio of Bcl-2 and Bax. The expression of Bcl-2 was significantly enhanced in the intact heart after 24 h of sildenafil treatment compared with control (P < 0.01, n = 3; Fig. 8, A and B). PD98059 completely blocked the increased expression of Bcl-2 with sildenafil. Although sildenafil reduced the expression of Bax compared with control (Fig. 8, A and C), the ratio of Bcl-2 to Bax was significantly increased following 24 h sildenafil treatment (P < 0.01, n = 3), which was diminished by cotreatment with PD98059 (Fig. 8, A and D).

DISCUSSION

Previous studies have shown that ERK, one of the members of MAPK family, is a signaling molecule in the cell survival or defense networks. It has been demonstrated that ERK protects against ischemia-reperfusion injury by reducing myocyte apoptosis and infarct size (22, 28, 32). A number of pharmacological agents including adenosine, bradykinin, opioid receptor agonists, and resveratrol trigger a preconditioning-like survival signaling by activating ERK (1, 7, 10, 30). ERK is also known to activate p90 ribosomal S6 kinase and upregulates cAMP response element-binding protein, phosphorylation of BCL-XL/Bcl-2-associated protein (BAD; Ser112), which suppress BAD-mediated apoptosis (13, 21). The primary goal of this study was to elucidate a causative role of ERK in the early and/or delayed phase of sildenafil-induced cardioprotection and to further delineate the relationship between ERK and other key mediators in sildenafil-induced delayed protection. The present study reports a number of interesting novel findings related to the signaling pathway by which sildenafil induces cardioprotection. We have shown here for the first time that ERK inhibitor PD98059 completely abolished sildenafil-induced early and delayed infarct-limiting cardioprotective effect (Fig. 2), and PD98059 also diminished the enhanced ERK phosphorylation by sildenafil (Fig. 4). Only a very recent study in isolated porcine retinal arterioles suggested that ERK signaling may lead to NO production and subsequent guanylyl cyclase activation and ATP-sensitive K⁺ channel opening, which mediates the vasodilatory action of sildenafil in retinal arterioles (31). The sildenafil-induced cardioprotection is not mediated by p38 MAPK because the putative inhibitor of this kinase, SB203580, failed to block sildenafil-induced delayed cardioprotection as reported previously (18). Furthermore, we found no evidence for p38 MAPK activation following acute exposure of cardioprotective doses of sildenafil in either whole heart or cultured cardiomyocytes (6). Another salient finding of the present study is that ERK phosphorylation occurs downstream of PKG within the sildenafil-triggered signaling cascade. This was demonstrated by failure of PD98059 to block sildenafil-induced phosphorylation of VASP protein, a putative marker of endogenous PKG activity (Fig. 5), whereas sildenafil-induced ERK activation was dependent on PKG, as reported recently (6).

It is notable that the infarct-limiting protection was stronger in the delayed phase than the early phase (Fig. 2). In the early phase of sildenafil-induced cardioprotection, the drug (1 μM) was infused for 10 min to isolated perfused hearts, which were immediately subjected to global ischemia-reperfusion. However, in the delayed phase, sildenafil was given intraperitoneally (0.71 mg/kg) 24 h before the same ischemia-reperfusion protocol. It is possible that during the prolonged (24 h) in vivo exposure to sildenafil, there may be de novo synthesis of multiple cardioprotective proteins and/or activation of additional signaling molecules, which are not present in the early phase due to shorter drug exposure time, i.e., 10 min. These
additional cardioprotective factors could have resulted in more pronounced reduction of infarct size in the delayed phase. Moreover, the routes of administration of sildenafil in the two phases were also different, which may be another reason for the difference in infarct size reduction between the two phases.

The present study also revealed for the first time that ERK mediates sildenafil-induced delayed cardioprotection via induction of the downstream cytoprotective proteins (Fig. 9), including iNOS/eNOS (Fig. 6) and Bcl-2 (Fig. 8) as well as phosphorylation of GSK-3β (Fig. 7). Numerous studies have documented a close interaction between NO and ERK; however, the cross talk between these two signaling molecules in either direction has not been confirmed. It has been reported that NO stimulated ERK1/2 via Ras activation or MAP kinase phosphastrate-3 inactivation (13). Xu et al. (28) also reported that the anti-infarct effect of NO donor S-nitroso-N-acetyl penicillamine was mediated by NOS-induced phosphorylation and activation of ERK1/2 in isolated rat cardiomyocytes and the intact heart, suggesting that ERK activation was downstream of NOS (28). However, other studies demonstrated that ERK activation is required for the induction of NOS (3, 24). Philipp et al. (16) also reported that ERK phosphorylation occurred upstream of NOS in acetylcholine-induced protection in the rabbit heart. ERK has been reported to be involved in serum phosphorylation of signal transducer and activators of transcription 1 and 3 (STAT1 and STAT3) (9, 26). Ischemic preconditioning induced serum phosphorylation of STAT1 and STAT3 by activating the ERK signaling pathway (29). A previous study suggested that ischemic preconditioning up-regulated cardiac iNOS by the combinatorial activation of STAT1/3 and ERK1/2 by resveratrol through p38 mitogen-activated protein kinase/mitogen- and stress-activated protein kinase (30). Moreover, persistent activation of STAT1/3 and/or NF-κB in sildenafil-induced delayed cardioprotection, we can logically speculate a possible participation of these cardioprotective signaling mediators. Further studies are needed to prove this assumption.

GSK-3β is an enzyme that regulates glycogen synthesis in response to insulin. Activation of GSK-3β has been shown to induce myocardial apoptosis and becomes inactivated when phosphorylated at Ser9 by activated Akt. A recent study showed that overexpression of GSK-3β-DN in cardiac myocytes inhibited TNF-α-induced apoptosis. Moreover, persistent inhibition of GSK-3β induced compensatory hypertrophy, inhibited apoptosis and fibrosis, and increased cardiac contractility in mice (11). In the present study, we observed ERK-dependent phosphorylation of GSK-3β, which persisted even 24 h after the sildenafil treatment (Fig. 6). To our knowledge, there is currently no cardioprotective drug that has been shown to have such a prolonged inhibitory effect on GSK-3β. This finding also suggests that a single dose of sildenafil may have a long-lasting protective effect against ischemia-reperfusion injury. In fact, unpublished results from our laboratory have shown that sildenafil-induced protective effect against ischemia-reperfusion injury could be captured even 7 days post-treatment in rabbit.

In conclusion, the present study has provided first direct evidence for the cause-and-effect relationship between sildenafil-induced anti-ischemic cardioprotection and phosphorylation of ERK. We have also reported that ERK mediates sildenafil-induced upregulation of iNOS/eNOS, Bcl-2, and the inactivation of GSK-3β. Such advances in understanding the cellular and molecular mechanisms of cardioprotection induced by a class of PDE-5 inhibitors will help in expanding the eventual utility of these compounds for other cardiovascular diseases in addition to the current use for treatment of erectile dysfunction and pulmonary artery hypertension.

REFERENCES


