Protein kinase C plays an essential role in sildenafil-induced cardioprotection in rabbits

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Das, Anindita, Ramzi Ockaili, Fadi Salloum, and Rakesh C. Kukreja. Protein kinase C plays an essential role in sildenafil-induced cardioprotection in rabbits. Am J Physiol Heart Circ Physiol 286: H1455–H1460, 2004; 10.1152/ajpheart.01040.2003.—Sildenafil citrate (Viagra) is the most widely used pharmacological drug for treating erectile dysfunction in men. It has potent cardioprotective effects against ischemia-reperfusion injury via nitric oxide and opening of mitochondrial ATP-sensitive K⁺ channels. We further investigated the role of protein kinase C (PKC)-dependent signaling pathway in sildenafil-induced cardioprotection. Rabbits were treated (orally) with sildenafil citrate (1.4 mg/kg) 30 min before index ischemia for 30 min and reperfusion for 3 h. The PKC inhibitor chelerythrine (5 mg/kg iv) was given 5 min before sildenafil. Infarct size (% of risk area) reduced from 33.65 ± 2.17 in the vehicle (saline) group to 15.07 ± 0.63 in sildenafil-treated groups, a 45% reduction compared with vehicle (mean ± SE, P < 0.05). Chelerythrine abolished sildenafil-induced protection, as demonstrated by increase in infarct size to 31.14 ± 2.4 (P < 0.05). Chelerythrine alone had an infarct size of 33.5 ± 2.5, which was not significantly different compared with DMSO-treated group (36.8 ± 1.7, P > 0.05). Western blot analysis demonstrated translocation of PKC-α, -θ, and -δ isoforms from cytosol to membrane after treatment with sildenafil. However, no change in the PKC-β and -ε isoforms was observed. These data provide direct evidence of an essential role of PKC, and potentially PKC-α, -θ, and -δ, in sildenafil-induced cardioprotection in the rabbit heart.

chelerythrine

SILDENAFIL CITRATE (Viagra), a specific phosphodiesterase-5 (PDE-5) inhibitor currently approved for the treatment of erectile dysfunction in men, has been shown to acutely enhance endothelium-dependent vasodilation in patients with heart failure (7). This drug allows accumulation of the potent relaxing agent cGMP in vascular smooth muscle cells by preventing its breakdown by PDE-5. Sildenafil has been shown to enhance nitric oxide (NO)-driven cGMP accumulation in the corpus cavernosum of rabbits without affecting cAMP formation (33). It causes a mild to moderate decrease in systolic and diastolic pressure because of the inhibition of PDE-5 in smooth muscle cells in the vascular bed (13). Interestingly, our laboratory (23) recently observed that sildenafil induced both acute and delayed cardioprotective effects against ischemia-reperfusion injury in rabbits, which was dependent on the opening of mitochondrial ATP-sensitive K⁺ (mitoKATP) channels. In addition, we demonstrated that sildenafil induced delayed preconditioning in the mouse heart through increased expression of inducible NO synthase (31). However, the signaling mechanism involved in cardioprotection is unknown.

Several studies (1, 2, 16, 17, 26, 37) suggest that protein kinase C (PKC) activation plays an important role in the mechanism of preconditioning. The PKC family of isozymes has three major subgroups: the conventional, calcium dependent (α, βI, βII, and γ); the novel; and the calcium independent (δ, ε, η, θ, and possibly μ). Translocation of PKC isoform(s) from the cytosolic to the particulate fraction (including sarcoplasmic, mitochondrial, as well as nuclear fractions) results in it binding to specific receptors of activated C kinase localized in membranes (20, 21). Translocated-specific PKC isoforms are believed to participate in several functions including the opening of mitoKATP channels or the induction of gene expression (9, 34, 36). It has been shown that PKC-mediated cardioprotection is isoform specific: the ε- and η-isoforms play an essential role in the development of ischemic preconditioning in rabbit myocardium (4, 15, 16, 27, 29). In addition, several pharmacological agents cause cardioprotective effect through selective translocation of PKC isoforms to the membrane fractions (14, 32, 40). However, the role of PKC or translocation of specific isoform(s) after sildenafil-induced cardioprotection is not known. Accordingly, the goal of the present investigation was to show 1) whether the cardioprotective effects of sildenafil are blocked by the PKC inhibitor chelerythrine in the rabbit heart and 2) to determine whether sildenafil induces translocation of specific PKC isoform(s) from the cytosolic to the particulate fraction.

MATERIALS AND METHODS

Animals. Male New Zealand White rabbits (2.8–3.3 kg) were used for the studies. The rabbits were supplied by the Blue and Gray Rabbitry (Unionville Lane, VA). The animals were allowed to readjust to the new housing environment for at least a week before the experiment. Standard food and water were freely accessible for the rabbits. Animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Experimental protocol. All animals were subjected to an infarction protocol consisting of 30 min of sustained ischemia by occlusion of the coronary artery, followed by 180 min of reperfusion (Fig. 1A). The effect of sildenafil was studied in the absence or presence of chelerythrine chloride. The myocardial infarction protocol was carried out 60 min after treatment with sildenafil. The rabbits were randomly assigned into one of the following groups. In group 1 (saline control, n = 6), rabbits received 0.9% saline. In group 2 (sildenafil, n = 6), the Viagra tablets were crushed, dissolved in water, and given orally to the rabbits. Because there is 40% bioavailability of sildenafil citrate...
Role of PKC in Sildenafil and Preconditioning

A

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Sildenafil, (Oral)(1.4 mg/kg)

60 min 30 min 60 min 120 min

Fig. 1. Experimental protocols showing infarct study after oral administration of sildenafil citrate (Sil) and/or intravenous administration of protein kinase C (PKC) inhibitor chelerythrine (Che) (A) and PKC measurement after oral administration of sildenafil (B).

After oral administration, we used double the dose of the intravenous route; i.e., 1.4 mg/kg, which is equivalent to clinical dose of 100 mg for a 70-kg patient. Our previous study (23) has shown that an oral dose of 1.4 mg/kg is as potent as the intravenous dose of 0.7 mg/kg in cardioprotection. In addition, a group of animals were given sildenafil citrate (0.7 mg/kg) as an intravenous bolus; the clinical dose of 50 mg administered to a 70-kg human patient (28). In group 3 (sildenafil + chelerythrine), chelerythrine chloride was dissolved in DMSO (25 mg/2.5 ml DMSO) and 5 mg/kg chelerythrine was administered 5 min before sildenafil treatment. In group 4 (chelerythrine), chelerythrine was given alone without sildenafil. In group 3 (DMSO), the solvent for chelerythrine, DMSO, was administered 35 min before ischemia-reperfusion protocol.

Surgical preparation. The rabbit model of ischemia-reperfusion protocol has been described previously (23). After the rabbits were anesthetized with ketamine HCl (35 mg/kg) and xylazine (5 mg/kg), a left thoracotomy was performed to expose the heart. Myocardial ischemia was induced by occlusion of coronary artery for 30 min, followed by reperfusion for 3 h. After completion of ischemia-reperfusion protocol, 500 IU of heparin were injected and the heart was quickly removed and mounted on a Langendorff apparatus. The coronary arteries were perfused with 0.9% NaCl containing 2.5 mM CaCl₂. After the blood was washed out, the ligation around the coronary artery was retightened and ~2 ml of 10% Evans blue dye were injected as a bolus into the aorta until most of the heart turned blue. The heart was perfused with saline to wash out the excess Evans blue. Finally, the heart was removed, frozen, and cut into 8–10 transverse slices from apex to base with equal thickness (~1 mm). The slices were then incubated in a 1% triphenyltetrazolium chloride solution in an isotonic phosphate buffer (pH 7.4) at 37°C for 30 min. The areas of infarcted tissue, the risk zone, and the whole left ventricle were determined by computer morphometry using a Bioquant imaging software. Infarct size was expressed both as a percentage of the left ventricle and ischemic risk area.

Measurement of hemodynamics. Hemodynamic measurements included heart rate and mean arterial pressure (MAP). Rate-pressure product (RPP) was calculated as the product of heart rate and peak arterial pressure.

Measurement of subcellular distribution of PKC isozymes. For measurement of PKC, rabbits were administered sildenafil citrate (1.4 mg/kg) or saline orally and hearts were excised 30, 60, and 120 min later (Fig. 1B). The left ventricle was dissected and stored in liquid nitrogen until used. The frozen tissue samples were ground in a prechilled mortar and pestle under liquid nitrogen. Total cellular proteins were obtained by glass-glass homogenization of the powdered tissue in 3 ml extraction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 50 μg/ml PMSF, protease inhibitor cocktail (10 μl/ml; product no. P8340, Sigma), and 0.3% β-mercaptoethanol, as described by Qiu et al. (29). The homogenates were centrifuged at 45,000 g for 30 min at 4°C. The supernatant containing cytosolic protein was saved, and the pellet was resuspended in 3 ml of same extraction buffer along with 1% (vol/vol) Triton X-100 and incubated on ice for 1 h and centrifuged at 45,000 g for 30 min to obtain supernatant (particulate fraction). The protein concentration was determined with the use of a protein assay kit (Bio-Rad; Hercules, CA). Cytosolic and membrane fractions (100 μg protein) were separated by SDS-PAGE on 10% denaturing acrylamide gels and transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk

Fig. 2. Bar diagrams infarct size (% risk area) (A) and risk area (illustrating % left ventricle (LV) (B)) after oral administration of sildenafil citrate and/or intravenous administration of PKC inhibitor chelerythrine after ischemia and reperfusion. Saline control, rabbits received 0.9% saline. Sil, rabbit received 1.4 mg/kg sildenafil 60 min before ischemia-reperfusion. Sil + Che, chelerythrine was administered 5 min before sildenafil treatment. Che, chelerythrine was given alone 65 min before ischemia-reperfusion. DMSO, the solvent for chelerythrine, DMSO, was administered 35 min before ischemia-reperfusion protocol. Results are means ± SE in 6 rabbits in each group. *P < 0.05 compared with saline control, sildenafil, sildenafil + chelerythrine, chelerythrine, and DMSO.

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milk in a buffer composed of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1% Tween 20 for 1 h. The blots were then incubated with 1,000-fold diluted primary antibodies against respective PKCs (Santa Cruz Biotechnology, Santa Cruz, CA) [cPKCα (SC-8393); cPKCβ1, (SC-8049); nPKCα (SC 8402); nPKCε (SC-1681); and nPKCθ (SC-1680)] to assess the expression of individual PKC isoform. To normalize for loading of protein, we used β-actin antibody (Sigma, A-2172). After 2 h of incubation with respective primary antibodies, the blots were washed and incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution, Amersham Pharmacia Biotech) for 1 h. The blots were developed with the use of a chemiluminescent system (ECL kit, Amersham). Each immunoblotting experiment was repeated twice and the results were averaged. To quantify the protein translocation, the optical density for each blot was scanned and analyzed with a densitometric system (Bioquant 98).

Data analysis and statistics. Data are presented as means ± SE. The difference among the treatment groups (for infarct size and hemodynamics) or the time points after sildenafil injection (for protein translocation) was compared with unpaired t-test or one-way ANOVA, followed by a Student-Newman-Keuls post hoc test. P < 0.05 was considered as statistically significant.

RESULTS

Infarct size. Figure 2A shows infarct size expressed as the percentage of risk area. Preconditioning with sildenafil resulted in a significant decrease in the infarct size from 33.6 ± 2.17 in the control (saline) group to 15.07 ± 0.63 in the sildenafil-treated rabbits, a 45% reduction compared with vehicle (means ± SE, P < 0.05). The infarct size increased significantly to 31.14 ± 2.4, (P < 0.05) with chelerythrine when given 5 min before sildenafil treatment. Chelerythrine alone had an infarct size of 33.5 ± 2.5, which was not significantly different compared with the DMSO (solvent of chelerythrine)-treated group (36.9 ± 2.3). A similar trend in the changes in infarct size was observed when expressed as a percentage of the left ventricle (not shown). Similarly, the differences between the risk areas expressed as a percentage of the left ventricle were not statistically significant between the groups (Fig. 2B). These data suggest that changes in the infarct size observed among various groups were not related to the percentage of the area of the left ventricle that was occluded by our technique. The infarct size (% of risk area) was reduced by 68% when sildenafil was administered intravenously (from 33.8 ± 1.7 in the saline-treated control group to 10.8 ± 0.9 in the sildenafil-treated rabbits). This infarct size reduction was blocked by chelerythrine.

Hemodynamics. Heart rate, MAP, and RPP are shown in Table 1. Heart rate, MAP, and RPP remained reasonably stable throughout the experimental period, although they gradually decreased in most of the groups. Except at the indicated time points, the mean values were not significantly different between the groups at any time point within the groups.

Translocation of PKC isoforms. We examined the subcellular distribution of five PKC isoforms (α, β1, δ, ε, and θ) after 30, 60, and 120 min of oral administration of sildenafil by Western blot with the use of isofrom-specific antibodies. As shown in Fig. 3, the PKC isoforms α, β1, δ, θ, and ε were expressed in both cytosolic and membrane fractions. The subcellular distribution of these isoforms was generally higher in the cytosol compared with the membrane fraction. No significant change of the translocation of PKC-α occurred from cytosol to membrane after 30 min of sildenafil treatment compared with control (saline treatment) (Fig. 3A). However, an increase in membrane PKC-α was observed at 60 min,

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Values are means ± SE; n = 6 rabbits/group. HR, heart rate (beats/min); MAP, mean arterial blood pressure (mmHg); RPP, rate-pressure product (mmHg). *P < 0.05 vs. sildenafil; **P < 0.05 vs. sildenafil + chelerythrine; †P < 0.05 vs. baseline; ‡P < 0.05 vs. preischemia; §P < 0.05 vs. 30-min ischemia.
which reached significantly higher levels by 120 min of sildenafl treatment. PKC-θ and -δ exhibited significant increase of the translocation in the membrane fraction after 30 to 120 min of sildenafl administration compared with control (Fig. 3, B and C). Quantitative analysis showed increase of PKC-α-to-β-actin ratio in the membrane fraction by sildenafl from 0.52 ± 0.06 (in control) to 0.70 ± 0.04 after 120 min ($P < 0.05$) (Fig. 3A). The ratio of PKC-θ-to-β-actin in the membrane fraction increased from 0.62 ± 0.06 in saline control to 0.87 ± 0.08 ($P < 0.05$) after 120 min of sildenafl treatment (Fig. 3B). Similarly, PKC-δ-to-β-actin ratio in the membrane fraction increased from 0.74 ± 0.06 (control) to 0.94 ± 0.03 (sildenafl) ($P < 0.05$) by 120 min of sildenafl treatment (Fig. 3C). In contrast, no significant increase of translocation of PKC-β (Fig. 3D) and PKC-ε (Fig. 3E) to membrane occurred after sildenafl treatment.

**DISCUSSION**

The use of sildenafl for treatment of erectile dysfunction by many patients with cardiovascular disease has resulted in a tremendous interest in the cardiovascular properties of the drug (3). Recently, we (23) reported that administration of sildenafl-induced cardioprotection as indicated by significant reduction in the infarct size compared with controls. In addition, we (31) showed that sildenafl-induced delayed cardioprotective effect in the mouse heart through upregulation of inducible and endothelial NO synthase. The hypothesis behind these studies was that vasodilatory action of sildenafl could potentially release endogenous mediators of preconditioning such as adenosine, bradykinin, or NO. One or more of these mediators may trigger signaling cascade leading to opening of the mitoK$_{ATP}$ channel resulting in acute and delayed cardioprotective effects.
There is substantial experimental evidence that one of the major intracellular signal transduction pathways controlling cardiac protection by ischemic preconditioning involves activation of PKC (24, 35). The question posed in this study was whether PKC plays a pivotal role in sildenafil-induced cardioprotection and to identify the specific isoform(s) may be involved in the protective role of sildenafil. Our data show that translocation of PKC-α and -δ started to increase after 30 min of sildenafil treatment, reaching to a significantly increased level after 120 min of sildenafil treatment, which coincided with the ischemia protocol performed 60 min after oral administration of sildenafil. Although statistically nonsignificant, there was a well-defined trend toward an increase in the translocations of PKC-α, -θ and -δ with respect to control at 30 and 60 min after sildenafil treatment. Our data shows that administration of chelerythrine, the blocker of PKC, before sildenafil abolished the infarct-limiting effect of sildenafil after ischemia-reperfusion. The reduction in infarct size was not altered by vehicle (i.e., DMSO) confirming that the blockade of protection was indeed due to chelerythrine only. Furthermore, chelerythrine did not have significant effect on infarct size in the sham ischemia-reperfused rabbit hearts, suggesting that the PKC antagonist interceded the signal transduction cascade during sildenafil treatment only. These data strongly indicate that PKC activation plays an important role in the signaling mechanisms leading to sildenafil-dependent cardiac protection in the rabbit heart.

Endogenous PKC exists in various isoforms with specific tissue distribution and sensitivity. Translocation of PKC from cytosolic to particulate compartments is commonly used as an index of PKC activation, which is not only limited to sarcolemmal membrane but also to cytoskeletal, mitochondrial, and nuclear fractions. In the present study, we identified and quantified different isoforms of PKC from cytosol and membrane fractions. Our results show significant translocation of PKC-α after 60 min of treatment with sildenafil. PKC-α is the major calcium-dependent PKC isoform located in the soluble fraction in resting neonatal cardiac myocytes and an increase in calcium concentration selectively translocates PKC-α to the particulate fraction (30). The activation of PKC-α is essential for the regulation of the Raf-Ras-Erk cascade by insulin-like growth factor 1 (25) or by hypertrophic signaling in adult rat cardiomyocytes (10). Whereas PKC-α, -δ, and -ε translocation has been studied in various models of ischemic and pharmacological preconditioning, the role of PKC-θ in cardioprotection remains unknown. PKC-θ is present in T lymphocytes as well as skeletal muscle, and its role in T cell signaling has been studied extensively (11). In the primary myotubes from neonatal mouse hindlimb muscle, a rapid translocation of PKC-θ to the membrane in response to treatment with the cholinergic receptor agonists, carbachol has been demonstrated (12). It was suggested that recruitment of PKC-θ to the membranes of myotubes after carbachol treatment plays a role in modulation of the function of membrane proteins, including receptors. Carbachol and PMA also caused an increase in PKC-α levels in the cytosol, followed by its increase in membrane fractions (12). Inagaki et al. (6) showed that a novel drug, JTV519, has protective effect against Ca2⁺ overload-induced myocardial injury and provides an anti-ischemic effect via specific activation of PKC-δ in rat hearts. Miyawaki and Ashraf (18) demonstrated that high-calcium preconditioning evoked the translocation of PKC-α and PKC-δ to the cell membrane. In addition, PKC-ε was translocated to the intercalated disk and suggesting that PKC-ε may modulate myocardial function through cell-to-cell interactions. Kawamura et al. (8) showed that ischemic preconditioning translocates PKC-α, -δ and -ε, but not PKC-δ, suggesting that the translocation of calcium-independent PKC-δ is essential for mediating ischemic preconditioning. The difference of the preconditioning procedures may also influence the importance of the PKC isoforms in mediating the protective effect (8). Pathophysiological stimuli, including heat shock and a combination of heat shock and ischemic preconditioning, also resulted in the translocation of PKC-α and -δ in young rats (5), further supporting the importance of these isoforms for the signaling cascade in cardioprotection. The downstream targets of PKC-mediated cardioprotection involve multiple signaling pathways, which include activation of MAPKs, e.g., ERK1/2 and p38 kinases (19, 22, 26, 38, 41). Recently, we demonstrated an essential role of PKC-δ in the delayed cardioprotection triggered by stimulation of adenosine A1 receptor subtype in the mouse (40). In these studies, the selective early translocation of PKC-δ in the membrane fractions initiated downstream signaling involving activation of transcription factor nuclear factor-κB, generation of NO, and opening of the mitoKATP channels (39), the possible mediators of delayed pharmacological preconditioning in the heart.

In conclusion, for the first time we have demonstrated that sildenafil-induced cardioprotection is dependent on activation of PKC. Our results also show that selective translocation of three PKC isoforms (i.e., α, δ, and θ) from cytosol to membrane fractions suggesting their potential role in sildenafil-induced cardioprotection. Further investigations are needed to determine the cause and effect of each of the translocated PKC isozymes in sildenafil-induced cardioprotection and to understand the mechanism(s) by which translocated isozymes orchestrate downstream targets involved in attenuation of ischemic injury. These studies would help in expanding our knowledge on using this drug for protection of ischemic myocardium in humans.

GRANTS
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