Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular and Cellular Cardiology 44 (2008) 105–113
Journal of Molecular and Cellular Cardiology
www.elsevier.com/locate/yjmcc

Original article

Essential role of mitochondrial Ca^{2+}-activated and ATP-sensitive K^+ channels in sildenafil-induced late cardioprotection

Xiaoyin Wang, Patrick W. Fisher, Lei Xi, Rakesh C. Kukreja *

Division of Cardiology, Department of Internal Medicine, Box 980204, Virginia Commonwealth University, 1101 East Mashall Street, Room 7-046, Richmond, VA 23298-0204, USA

Received 5 September 2007; received in revised form 2 October 2007; accepted 7 October 2007
Available online 16 October 2007

Abstract

Sildenafil (Viagra), a phosphodiesterase type-5 inhibitor used in treatment of male erectile dysfunction and pulmonary hypertension can induce cardioprotection through opening of mitochondrial ATP-sensitive K^+ channels (mitoK_{ATP}). Recent studies suggest that activation of mitochondrial Ca^{2+}-activated K^+ channels (mitoK_{Ca}) also has anti-ischemic effects. However, the relative role of mitoK_{Ca} and mitoK_{ATP} in sildenafil-induced cardioprotection remains unknown. In the present study, adult male ICR mice were pretreated with sildenafil (0.71 mg/kg, *i.p.*) 24 h prior to 20 min of global ischemia followed by 30 min of reperfusion in Langendorff mode. Paxilline (blocker of K_{Ca}) or 5-hydroxydecanoic acid (5-HD; blocker of mitoK_{ATP}) was administered either 30 min before sildenafil or 10 min prior to ischemia. Treatment with sildenafil reduced infarct size, which was abolished by either paxilline or 5-HD. Furthermore, *in vivo* gene knockdown of β1 subunit of K_{Ca} (K_{Ca}-β1) using small interfering RNA (siRNA) administered 48 h before sildenafil injection blocked the infarct limiting effect of sildenafil. The protective effect of sildenafil was preserved in mice treated with non-target siRNA. Western blots demonstrated selective protein expression of K_{Ca}-β1 in cardiac mitochondria and the gene knockdown effect of siRNA on K_{Ca}-β1. The level of K_{Ca}-β1 protein was not upregulated following treatment with sildenafil. We conclude that both mitoK_{Ca} and mitoK_{ATP} play a critical role in triggering and mediating sildenafil-induced delayed cardioprotection. The results suggest that activation of mitoK_{Ca} and mitoK_{ATP} are crucial for maintaining mitochondrial homeostasis and reducing cell death in sildenafil-induced preconditioning against ischemia-reperfusion injury.

Keywords: Phosphodiesterase inhibitor; Potassium channel; Ischemia-reperfusion; Cardioprotection; Small interfering RNA

Sildenafil citrate (Viagra™) is the first phosphodiesterase type-5 (PDE-5) inhibitor approved by the U.S. Federal Drug Administration for the treatment of patients with erectile dysfunction [1] and pulmonary hypertension [2]. Our laboratory first demonstrated that sildenafil induced myocardial protection via triggering a complex cell signaling cascade that involved nitric oxide synthases and mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}) [3–5] leading to preservation of mitochondrial membrane potential and alleviating necrotic as well as apoptotic cell death caused by ischemia-reperfusion [6] or loss of myofibrillar integrity by cardiotoxicity of anti-cancer drug—doxorubicin [7].

Cardiac mitoK_{ATP} have long been recognized as mediators of protection against I/R injury in intact hearts [8–11] and isolated cardiomyocytes [12]. However, mitoK_{Ca} have recently been identified on the inner membrane of cardiac mitochondria [13] and have become an emerging targets for anti-ischemic cardioprotection [13–16] through mechanism similar to mitoK_{ATP} involving enhanced uptake of mitochondrial K^+ and reduced Ca^{2+} overload in cardiomyocytes [13]. We and others have demonstrated that NS-1619, a putative K_{Ca} channel opener, reduced infarct size acutely [13–16] as well as 24 h later [16] following treatment with this drug. The protective effect of NS-1619 was shown to be abolished by paxilline [13,14,16], a specific blocker of K_{Ca}.

Although K_{Ca} are abundantly expressed in vascular smooth muscle cells, they are also expressed broadly in other cell types and have functional roles in neurons, kidney, skeletal muscle, and secretory cells [17,18]. In contrast to the aforementioned

* Corresponding author. Tel.: +1 804 828 0389; fax: +1 804 828 8700.
E-mail address: rakesh@vcu.edu (R.C. Kukreja).

0022-2828/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.yjmcc.2007.10.006

tissues where K_{Ca} are found in abundance in the plasma membrane, they are specifically located on the inner mitochondrial membrane in cardiomyocytes [13,19]. Structurally, K_{Ca} channels are composed of two dissimilar subunits, α and β [17,20,21]. The functional roles of K_{Ca} channels are in part associated with accessory β-subunits, which play an important role in modulating Ca^{2+} signaling *in vivo* [17,22]. The cytoprotective effect of the β1 subunit of K_{Ca} is thought to be, in part, due to its ability in altering calcium sensitivity [21,23], increasing channel open time, and counteracting the altered mitochondrial bioenergetics that ensue from ischemic insult. Furthermore, it has been postulated that mitoK_{Ca} facilitate membrane hyperpolarization [24,25] and may in fact act in concert with the mitoK_{ATP} by providing additive protection against loss of mitochondrial membrane potential [13].

Despite the previously reported mitoK_{ATP} dependence of sildenafil-induced cardioprotection [4], it remains inexplicable whether this protection is also dependent upon activation of mitoK_{Ca}. Moreover, the relative contribution of mitoK_{ATP} and mitoK_{Ca} (particularly β1 subunits) for cardioprotection by sildenafil is unknown. Because sildenafil induces vasorelaxation via protein kinase G (PKG)-dependent activation of K_{Ca} in vascular smooth muscle [25–27], we hypothesized that this drug may activate mitoK_{Ca} in cardiomyocytes, which would potentially result in cardioprotection. To test this hypothesis, we performed the current investigation using our established mouse model of sildenafil-induced delayed cardioprotection [5]. We used selective blockers of K_{Ca} (i.e., paxilline) and mitoK_{ATP} (i.e., 5-hydroxydecanoic acid, 5-HD) to discern the role of these channels in cardioprotection. To conclusively determine the essential role of β1 subunit of mitoK_{Ca} in sildenafil-induced protection, we also employed small interfering RNA (siRNA) to selectively knockdown the expression of the protein [28,29].

1. Materials and methods

1.1. Animals, drugs, and experimental protocols

All animal experiments were performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, Revised 1996) and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

The pure solid powder of sildenafil was a gift from Pfizer, Inc. (New York, NY) and dissolved in saline. Paxilline was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA) and dissolved in DMSO. 5-HD was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in saline.

Adult male ICR mice were purchased from Harlan (Indianapolis, IN) and housed in a temperature-controlled room with 12:12 h of light:dark cycle. The diet consisted of normal rodent chow and water was *ad libitum*. As illustrated in Fig. 1A, mice ($n=6–7$ per group) were treated with sildenafil (0.71 mg/kg, *i.p.*; [5]) or volume-matched saline 24 h prior to 20 min of global ischemia followed by 30 min of reperfusion in a Langendorff isolated, Krebs buffer-perfused heart model [16,30]. Paxilline was administered either 30 min before sildenafil injection (50 μg/kg, *i.p.*); i.e., the "Trigger Phase" or via intracoronary infusion (1 μmol/L) for 10 min prior to global ischemia, i.e., the "Mediator Phase". The method of administration of 5-HD was similar to the abovementioned paxilline administration. An *i.p.* injection of 5-HD (1.2 mg/kg) was given at the Trigger Phase and 100 μM of 5-HD was intracoronarily infused for 10 min at the Mediator Phase.

As described previously [16], ventricular contractile force and heart rate were continuously recorded with a force-displacement transducer (Model FT03; Grass Technologies, West Warwick, RI) that was connected to a computerized data acquisition and analysis system (PowerLab 8SP; ADInstruments, Colorado Springs, CO). Coronary flow rate was measured by timed collection of the coronary effluent. Following ischemia-reperfusion, the hearts were frozen under −20 °C, sliced, and stained with 10% triphenyltetrazolium chloride (TTC; Sigma-Aldrich) for 30 min at room temperature and fixed in 10% formalin for at least 2 h before measuring infarct size (% of risk area) using computer morphometry (Bioquant 98; Bioquant Image Analysis Corp., Nashville, TN).

1.2. Source and delivery of siRNA

A chemically synthesized siRNA (siSTABLE™) was custom-made by Dharmacon RNA Technologies (Lafayette, CO). The siSTABLE™ is a modified siRNA with improved nuclease stability *in vivo* and enhanced silencing longevity. This modified siRNA was converted to 2′-hydroxyl, annealed and desalted duplexes. The targeted siRNA duplex (siRNA-K_{Ca}-β1) was designed for gene sequence of β1 subunit of K_{Ca} (GGUCAGAGCCAAUUUCUAU). A scramble siRNA duplex was also generated for serving as a non-target control (siRNA-NT). The sequence of siRNA-NT is UAGCGACUAAACACAUCAA. All siRNA duplexes were resuspended in RNase-free 1 × PBS for *i.p.* delivery. Animals in the siRNA-K_{Ca}-β1 and siRNA-NT groups were pretreated with the respective siRNA (4 mg/kg, *i.p.*) 48 h before the sildenafil treatment. Twenty-four hours following sildenafil treatment, the hearts were subjected to the same ischemia-reperfusion experiments in Langendorff mode (see Fig. 1B).

1.3. Isolation of mouse cardiac mitochondria

Mitochondria were isolated from mouse ventricular tissue by enzymatic digestion, homogenization, and differential centrifugation. Under surgical anesthesia with pentobarbital sodium (~120 mg/kg, *i.p.*; Abbott Laboratories, Chicago, IL), mouse heart was quickly removed from the thoracic cavity, the fresh ventricular tissue was excised and washed with a homogenization buffer supplied by Sigma-Aldrich (10 mmol/L HEPES, 200 mmol/L mannitol, 70 mmol/L sucrose, and 1 mmol/L EGTA, pH 7.5 at 4 °C). The tissue was finely minced and incubated in ice-cold homogenization buffer containing trypsin (0.25 mg/mL; Sigma-Aldrich) for 20 min, then diluted with albumin solution (Sigma-Aldrich) for a final concentration of 10 mg/mL. The minced tissue was resuspended and