Free Radical Biology and Medicine 60 (2013) 80–88

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Phosphodiesterase-5 inhibitor tadalafil attenuates oxidative stress and protects against myocardial ischemia/reperfusion injury in type 2 diabetic mice

Saisudha Koka, Anindita Das, Fadi N. Salloum, Rakesh C. Kukreja *

Pauley Heart Center, Division of Cardiology, Department of Internal Medicine, Virginia Commonwealth University Medical Center, Richmond, VA 23298-0204, USA

ARTICLE INFO

Article history:
Received 7 September 2012
Received in revised form
4 January 2013
Accepted 29 January 2013
Available online 4 February 2013

Keywords:
Phosphodiesterase-5 inhibitors
Type 2 diabetes
Myocardial I/R injury
ROS
NAD(P)H oxidase
cardioprotection
Free radicals

ABSTRACT

Diabetic patients exhibit increased risk for the development of cardiovascular diseases primarily because of impaired nitric oxide (NO) bioavailability. The phosphodiesterase-5 (PDE-5) inhibitor sildenafil restores NO signaling and protects against ischemia/reperfusion (I/R) injury. In this study, we determined the effect of the long-acting PDE-5 inhibitor tadalafil on diabetes-associated complications and its role in attenuating oxidative stress after I/R injury in type 2 diabetic db/db mice. Adult male db/db mice ($n=40$/group) were randomized to receive dimethyl sulfoxide (10% DMSO, 0.2 ml, ip) or tadalafil (1 mg/kg in 10% DMSO, ip) for 28 days. After 28 days treatment, the hearts were isolated and subjected to 30 min global ischemia followed by 60 min reperfusion in the Langendorff mode. Infarct size was measured using computer morphometry of tetrazolium-stained sections. Cardiomyocytes were isolated from a subset of hearts and subjected to 40 min simulated ischemia followed by 1 h of reoxygenation (SI/RO). Dichlorodihydrofluorescein diacetate and JC-1 staining was used to measure reactive oxygen species (ROS) generation and mitochondrial membrane potential ($\Delta\psi m$), respectively. Another subset of hearts was used for the estimation of lipid peroxidation, glutathione, and the expression of myocardial pRac1, Rac1, gp91^{phox}, p47^{phox}, and p67^{phox} by Western blot. Tadalafil treatment improved the metabolic status and reduced infarct size compared to the untreated db/db mice ($21.2 \pm 1.8\%$ vs $45.8 \pm 2.8\%$; $p < 0.01$). The db/db mice showed enhanced oxidative stress in cardiomyocytes as indicated by a significant increase in ROS production. Cardiac NAD(P)H oxidase activity, lipid peroxidation, and oxidized glutathione were also increased in db/db mice compared to nondiabetic control animals. Tadalafil treatment in db/db mice suppressed oxidative stress, attenuated myocardial expression of pRac1 and gp91^{phox}, and also preserved the loss of $\Delta\psi m$ in cardiomyocytes after SI/RO. In conclusion, these results demonstrate that chronic treatment with tadalafil attenuates oxidative stress and improves mitochondrial integrity while providing powerful cardioprotective effects in type 2 diabetes.

1. Introduction

Phosphodiesterase-5 (PDE-5) inhibitors, including sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis), are erectile dysfunction (ED) drugs that also reduce damage to the penile vascular endothelium by upregulating endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), and NO production [1–3]. PDE-5 inhibitors are commonly used among the diabetic patient population because ED is a major and prevalent vascular complication in diabetes. ED is present in 32% of insulin-dependent diabetics and 46% of non-insulin-dependent diabetics. In fact, the PDE-5 inhibitor sildenafil has become a first-line therapy for diabetic patients with ED [4]. Pioneering studies from our laboratory have shown that PDE-5 inhibitors trigger NO signaling and protect against myocardial ischemia/reperfusion (I/R) injury [5–8]. Several other investigators have also demonstrated the cardioprotective effects of PDE-5 inhibitors in various models of ischemic injury [9–11]. Moreover, PDE-5 inhibitors attenuate cardiac dysfunction after myocardial infarction and doxorubicin-induced cardiomyopathy [8,12–15].

An increased incidence of myocardial infarction and adverse outcomes following ischemic events has been reported in diabetic patients [16]. The cardiac diseases among diabetics are primarily due to impaired NO bioavailability leading to endothelial dysfunction, which plays an integral role in the development of sequelae of cardiovascular disorders [17]. Downregulation of the NO–cGMP pathway has been implicated in the pathogenesis of diabetes-induced cardiovascular complications [18,19]. Consistent with these observations, type 2 diabetic patients have

* Corresponding author. Fax: +804 8288700.
E-mail address: rakesh@vcu.edu (R.C. Kukreja).

0891-5849/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2013.01.031

impaired NO synthesis and decreased expression of eNOS and iNOS [20]. Recent studies suggest that the NO signaling pathway may regulate and promote muscle glucose uptake and enhance muscle glucose utilization as well [21,22]. Based on this background information, we contemplated that PDE-5 inhibitors would restore NO signaling in heart and cardiomyocytes [2,7] and might improve cardiovascular complications associated with type 2 diabetes. In support of this notion, previous reports demonstrated that sildenafil and vardenafil improved vasorelaxation through enhanced endogenous NO signaling in streptozotocin-induced diabetic rats [23,24]. Similarly, in a clinical study, chronic (alternate-day) administration of tadalafil in men with ED of any etiology led to improved endothelial function as indicated by marked changes in serum markers of endothelial function [25]. Furthermore, both acute and chronic administration of sildenafil improved endothelial function in patients with type 2 diabetes [26,27]. A recent study from our laboratory demonstrated that tadalafil reversed detrimental remodeling of myocardial proteins involved in cytoskeletal rearrangement and redox regulation in type 2 diabetic mice [28]. Moreover, tadalafil therapy in type 2 diabetic mice ameliorated circulating inflammatory cytokines and chemokines while improving fasting glucose levels and reducing infarct size after I/R injury in the heart [29]. However, the potential role of tadalafil in attenuating oxidative stress, including the generation of reactive oxygen species (ROS), lipid peroxidation, or maintenance of glutathione levels in the type 2 diabetic heart, is currently unknown. Notably, it is also unknown whether tadalafil modulates NAD(P)H oxidases, which are a major source of ROS generation in hypertension, atherosclerosis, and diabetes [30–33]. Based on this background, this study was designed to determine the effects of chronic treatment of tadalafil in type 2 diabetic mice on: (a) myocardial infarct size after I/R injury, (b) ROS generation and mitochondrial membrane potential ($\Delta\psi m$) in cardiomyocytes after simulated ischemia/reoxygenation injury in vitro, (c) myocardial NADPH oxidase activity as well as expression of the enzyme subunits, and (d) attenuation of myocardial oxidative stress. We chose tadalafil in this study because this drug may have a number of favorable characteristics for the treatment of diabetic conditions. For example, tadalafil is a long-acting PDE-5 inhibitor ($t_{1/2}=17.5$ h) compared to other PDE-5 inhibitors such as sildenafil and vardenafil, whose duration of action ranges between 4 and 8 h. The pharmacokinetic properties of tadalafil allow for the most sustained PDE-5 inhibition among this class of agents. Tadalafil is also relatively more specific for PDE-5 than other PDE isoforms [34]. In addition, tadalafil is the only PDE-5 inhibitor whose activity is unaffected by food. It has a relatively short time to the onset of action (16–17 min) and is more slowly metabolized than sildenafil; therefore, this drug can potentially be used at lower doses for long-term management in diabetic patients.

2. Material and methods

2.1. Animals and experimental protocol

Adult male diabetic db/db (BKS.Cg-Dock7^{m}+/+Lepr^{db}/J strain) mice and wild-type, nondiabetic control mice (C57BLKS/J background) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University. All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (No. 85-23, Revised 1996). The db/db mice ($n=40$/group) were randomized to receive dimethyl sulfoxide (10% DMSO, 0.2 ml, ip) or tadalafil (1 mg/kg in 10% DMSO, ip) daily for 28 days. The tadalafil dose (1 mg/kg bw) was chosen based on interspecies dose extrapolation scaling to result in plasma concentrations equivalent to those found in humans receiving an oral dose of 20 mg/day. Upon completion of 28 days therapy, the mice were sacrificed; blood was collected for assessment of biochemical parameters and the mouse hearts were collected for further experimental analysis.

2.2. Measurement of plasma glucose, triglycerides, and insulin

After the animals were sacrificed, blood was collected into heparinized tubes and centrifuged at 1000*g* for 10 min at 4 °C to collect plasma, which was stored at −80 °C. The plasma samples were assayed for glucose and triglycerides using commercially available colorimetric assay kits (Cayman Chemicals, Ann Arbor, MI, USA). Plasma insulin concentrations were determined by ELISA following the manufacturer's instructions (Crystal Chem, Downers Grove, IL, USA).

2.3. Global ischemia/reperfusion injury by Langendorff method

The methodology for the isolated perfused mouse heart preparation was described previously in detail [35,36]. In brief, the animal was anesthetized and the heart was quickly removed from the thorax and placed into a small dish containing ice-cold perfusate with heparin. The aortic opening of the heart was rapidly cannulated and tied on a 20-gauge blunt needle that was, in turn, connected to a Langendorff perfusion system. After the cannulation, the heart was retrogradely perfused at a constant pressure of 55 mm Hg with modified Krebs–Henseleit solution containing (in mM) 118 NaCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, and 0.5 EDTA. The perfusion solution was continuously gassed with 95% O_2–5% CO_2 (pH 7.34–7.49) and warmed by a heating/cooling bath. The heart temperature was continuously monitored and maintained at 37 °C throughout the experiment. After a 30-min stabilization period, the heart was subjected to a 30-min no-flow normothermic global ischemia by clamping the perfusion tubing connected to the aortic cannula. Reperfusion was started by opening the aortic cannula and continued for 60 min.

2.4. Measurement of infarct size

The heart was immediately removed at the end of reperfusion from the Langendorff apparatus, weighed, and frozen at −20 °C. The frozen heart was manually cut into seven or eight transverse slices of approximately equal thickness (~0.8 mm) and stained by incubation in 10 mM triphenyltetrazolium chloride (TTC) for 30 min at room temperature (22 °C). TTC buffer was then replaced with 10% formaldehyde, and the slices were fixed for 4–6 h before the infarct area and risk zone were measured using computer morphometry (Bioquant 98). The risk area was calculated as total ventricular area minus the area of cavities. The infarct size was calculated as a percentage of the risk area.

2.5. Isolation of ventricular cardiomyocytes

The ventricular cardiomyocytes were isolated using an enzymatic technique modified from the previously reported method [37]. In brief, the mice were anesthetized with pentobarbital sodium (100 mg/kg intraperitoneally), and the heart was quickly removed from the chest. Within 3 min, the aortic opening was cannulated onto a Langendorff perfusion system, and the heart was retrogradely perfused (37 °C) at a constant pressure of 55 mm Hg for 5 min with