Abstract

Sildenafil induces powerful cardioprotection against ischemia/reperfusion (I/R) injury. Since adenosine is known to be a major trigger of ischemic preconditioning, we hypothesized that A1 adenosine receptor (A1AR) activation plays a role in sildenafil-induced cardioprotective signaling. Adult male C57BL wild-type (WT) mice or their corresponding A1AR knockout (A1AR-KO) mice were treated intraperitoneally (i.p.) with either sildenafil (0.71 mg/kg, equivalent to 50 mg dose for a 70 kg patient) or volume-matched saline. The selective A1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.1 mg/kg, i.p.) was administered 30 min before sildenafil. The hearts were isolated 24 h later and subjected to 30 min of global ischemia and 1 h of reperfusion in Langendorff mode. Post-ischemic myocardial infarct size (mean±SEM; % of risk area) was reduced in C57BL-WT mice treated with sildenafil (5.6±0.9) versus saline control group (27.3±2.1; \( p < 0.05; n=6/each \)). However, sildenafil failed to protect the A1AR-KO hearts (31.6±1.9 vs. 32.3±1.5 with saline, \( p > 0.05 \)). Additionally, DPCPX treatment abolished the infarct limiting effect of sildenafil (27.3±3.2, \( p < 0.05 \)). DPCPX alone had no effect on infarct size as compared with the control group. No significant changes in post-ischemic recovery of left ventricular pressure and heart rate were observed in the sildenafil-treated group. We further examined the effect of sildenafil in protection against simulated ischemia and reoxygenation injury in adult cardiomyocytes derived from WT and A1AR-KO mice. WT myocytes treated with sildenafil (1 \( \mu \)M) demonstrated significantly lower trypan blue-positive necrotic cells. However, cardiomyocytes derived from A1AR-KO mice or DPCPX-treated WT cells failed to show protection against necrosis with sildenafil. These results suggest that A1AR activation following treatment with sildenafil plays an integral role in the signaling cascade responsible for delayed protection against global I/R injury.

Keywords: Phosphodiesterase inhibitors; Myocardial preconditioning; Ischemia–reperfusion injury; cGMP; Adenosine; Adenosine A1 receptor; Infarct size

1. Introduction

Phosphodiesterase-5 (PDE-5) inhibitors including sildenafil, vardenafil and tadalafil are a class of drugs that have been developed for treatment of erectile dysfunction (ED) [1] and more recently for pulmonary artery hypertension [2]. We first showed that sildenafil induced preconditioning-like protective effects against ischemia/reperfusion (I/R) injury in the rabbit [3] and mouse heart [4]. Moreover, sildenafil attenuated necrosis as well as apoptosis in adult cardiomyocytes subjected to simulated ischemia/reoxygenation (SI/RO) [5]. A series of studies from our laboratory further showed that activation of protein kinase C [6], phosphorylation of extracellular-signal-regulated kinase (ERK) [7], synthesis of inducible and endothelial nitric oxide synthases (iNOS/eNOS) [4] and opening of mitochondrial K\textsubscript{ATP} channels [3] played a crucial role in protection against I/R injury with sildenafil. More recently, we also showed that both sildenafil and vardenafil provided cardioprotection against I/R through opening of mitochondrial K\textsubscript{ATP} channels when these drugs were infused at the time of reperfusion in rabbits [8].

Adenosine has been shown to be an important mediator of acute and delayed cardiac preconditioning [9–11]. Adenosine receptor activation has been shown to protect the heart against...
reversible and irreversible ischemic injury in multiple species and preparations [12,13]. Moreover, A1AR knockout (A1AR-KO) mice completely abolished the cardioprotective effect of ischemic preconditioning [14]. Previous studies from our laboratory have also shown that A1AR activation induces delayed protection through induction of iNOS [15] and opening of KATP channel [11]. Considering several similarities between the signaling cascade triggered by A1AR activation and sildenafil-induced protection, we hypothesized that the infarct limiting effect of sildenafil might be mediated via activation of A1AR. We speculated that sildenafil pretreatment would mimic a delayed preconditioning-like effect potentially through increased A1AR expression or augmented adenosine release from cells into the interstitial fluid which would make adenosine available to interact with its receptors, particularly during ischemia. In the current investigation, we took advantage of the A1AR-KO mice and the selective A1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) to elucidate the direct cause-and-effect of A1AR in inducing delayed cardioprotection in mice following treatment with sildenafil.

2. Materials and methods

2.1. Animals

Adult male C57BL mice were supplied by The Jackson Laboratory (Bar Harbor, ME). The body weight for these animals ranged between 27.1 and 32.7 g. A1AR-KO mice were obtained from a sub-colony of the original lines generated by Sun et al. [16]. A1AR-KO mice were bred on site at the Virginia Commonwealth University School of Medicine. The deletion of A1AR gene in the knockout mice was confirmed by RT-PCR.

2.2. Langendorff isolated perfused heart preparation

The methodology of the Langendorff isolated perfused heart has been previously described in detail [17]. In brief, the mice were anesthetized with an i.p. injection of sodium pentobarbital (100 mg/kg containing 33 international units of heparin). The hearts were removed and submerged in ice-cold Krebs–Henseleit (K–H) solution containing heparin. The hearts were removed and submerged in ice-cold Krebs–Henseleit (K–H) solution containing heparin. The aorta was secured to a 20 gauge stainless steel blunt needle, and the hearts were perfused at a constant temperature and pressure in a retrograde fashion via the aorta with K–H solution. Following a stabilization period of 30 min, I/R injury was produced by subjecting the hearts to 30 min of normothermic, no-flow global ischemia followed by reperfusion for 60 min. To obtain the developed pressure, a left atrial incision was made to expose the mitral annulus through which a water-filled latex balloon was passed into the left ventricle (LV). The balloon was attached via polyethylene tubing to a Gould pressure transducer that was connected to a PowerLab Acquisition System (ADInstruments, Australia). The balloon was inflated to adjust the LV end-diastolic pressure (LVEDP) to ∼10 mm Hg. Myocardial ischemia damage was measured using multiple, independent end points of tissue injury. These included infarct size, LV developed pressure (LVDP), LVEDP, rate pressure product (RPP), heart rate, and coronary flow by timed collection of the perfusate. LVDP was calculated by subtracting LVEDP from the peak systolic pressure. RPP, an index of cardiac work, was calculated by multiplying LVDP with heart rate. The hearts were not paced.

2.3. Study protocol

Mice were randomly assigned into one of the following groups as shown in Fig. 1:

- **Group I** (saline control, C57BL (WT) mice; n = 6): WT mice received 0.2 ml 0.9% saline, i.p.;
- **Group II** (sildenafil, WT mice; n = 6): Pure sildenafil powder (kindly provided by Pfizer, Inc.) was dissolved in 0.2 ml saline. This preparation was given in WT mice as 0.71 mg/kg i.p. bolus, approximating, on a mg/kg basis, the clinical dose of 50 mg administered to a 70 kg patient;
- **Group III** (DPCPX+sildenafil, WT mice; n = 6): DPCPX (0.1 mg/kg) was administered 30 min prior to sildenafil treatment in WT mice as in Group II;
- **Group IV** (DPCPX+saline, WT mice; n = 6): WT mice were treated with DPCPX (0.1 mg/kg) 30 min prior to saline treatment as in Group I;
- **Group V** (DMSO+sildenafil, WT mice, n = 6): DMSO (solvent for DPCPX) was administered in WT mice 30 min prior to sildenafil treatment as in group II;
- **Group VI** (DMSO+saline, WT mice, n = 6): WT mice were given DMSO 30 min prior to saline treatment as in Group I;
- **Group VII** (saline control, A1AR-KO; n = 6): A1AR-KO mice received 0.2 ml 0.9% saline, i.p.;
- **Group VIII** (sildenafil, A1AR-KO; n = 6): A1AR-KO mice received 0.71 mg/kg sildenafil as in Group II.

Twenty-four hours later, the hearts were isolated and subjected to I/R as described above.

2.4. Isolation of adult mouse ventricular cardiomyocytes

Adult male C57BL-WT or A1AR-KO mice were used in this study. The ventricular cardiomyocytes were isolated using an enzymatic technique modified from the previously reported method [5,18,19]. In brief, the animal was anesthetized with pentobarbital sodium (100 mg/kg, i.p.) and the heart was quickly removed. 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 a Ca2+-free bicarbonate-based buffer containing (in mM): 120 NaCl, 5.4 KCl, 1.2 MgSO4, 1.2 NaH2PO4, 5.6 glucose, 20 NaHCO3, 10 2,3-butanediol monoxime, and 5 taurine, which was continuously bubbled with 95%O2+5%CO2. The enzymatic digestion was commenced by adding collagenase type II (Worthington, 0.5 mg/ml each) and protease type XIV (0.02 mg/ml) to the perfusion.
buffer and continued for \(\sim 15\) min. 50 \(\mu\)M Ca\(^{2+}\) was then added to the enzyme solution for perfusing the heart for another 10–15 min. The digested ventricular tissue was cut into chunks and gently aspirated with a transfer pipette for facilitating the cell dissociation. The cell pellet was resuspended for a 3-step Ca\(^{2+}\) restoration procedure (i.e., 125, 250, 500 \(\mu\)M Ca\(^{2+}\)). The freshly isolated cardiomyocytes were then suspended in minimal essential medium (Sigma catalogue\# 6 M1018, pH 7.35–7.45) containing 1.2 mM Ca\(^{2+}\), 12 mM NaHCO\(_3\), 2.5% fetal bovine serum, and 1% penicillin–streptomycin. The cells were then plated onto the 35 mm cell culture dishes, which were pre-coated with 20 \(\mu\)g/ml mouse laminin in PBS +1% penicillin–streptomycin for 1 h. The cardiomyocytes were cultured in the presence of 5% CO\(_2\) for 1 h in a humidified incubator at 37 °C, which allowed cardiomyocytes to attach to the dish surface prior to the experimental protocol.

2.5. Experimental protocol

The cultured cardiomyocytes were incubated under 37 °C and 5% CO\(_2\) for 1 h with or without 1 \(\mu\)M sildenafil powder dissolved in distilled water. The drug solution was filtered (0.45 \(\mu\)m pore size) before adding into the cell medium. Cardiomyocytes were subjected to simulated ischemia (SI) for 40 min by replacing the cell medium with an “ischemia buffer” which contained (in mM): 118 NaCl, 24 NaHCO\(_3\), 1.0 NaH\(_2\)PO\(_4\), 2.5 CaCl\(_2\)–2H\(_2\)O, 1.2 MgCl\(_2\), 20 sodium lactate, 16 KCl, 10 2-deoxyglucose (pH adjusted to 6.2). In addition, the cells were incubated under hypoxic conditions at 37 °C during the entire SI period by adjusting the tri-gas incubator to 1–2% O\(_2\) and 5% CO\(_2\). Reoxygenation (RO) was accomplished by replacing the ischemic buffer with normal medium under normoxic conditions. Assessment of cell necrosis was performed after 1 h of RO.

2.6. Evaluation of cell viability

Cell viability was assessed by trypan blue exclusion assay. At the end of protocol, 20 \(\mu\)L of 0.4% trypan blue (Sigma-Aldrich) was added into the culture dish. After \(\sim 5\) min of equilibration, the cells were counted under microscope.

### Table 1
Baseline cardiac functional parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>HR bpm</th>
<th>LVEDP mm Hg</th>
<th>LVDP mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT control</td>
<td>404±5</td>
<td>7.5±0.6</td>
<td>97.7±3.1</td>
</tr>
<tr>
<td>WT sildenafil</td>
<td>403±5</td>
<td>8.1±0.4</td>
<td>110.3±4.0</td>
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<tr>
<td>DPCPX+ sildenafil</td>
<td>395±5</td>
<td>7.1±0.4</td>
<td>97.7±2.9</td>
</tr>
<tr>
<td>DPCPX+ saline</td>
<td>408±7</td>
<td>7.7±0.4</td>
<td>96.6±4.5</td>
</tr>
<tr>
<td>DMSO+ sildenafil</td>
<td>391±4</td>
<td>7.7±0.5</td>
<td>107±3.1</td>
</tr>
<tr>
<td>DMSO+ saline</td>
<td>399±3</td>
<td>7.5±0.5</td>
<td>102.4±2.5</td>
</tr>
<tr>
<td>A1AR-KO+ saline</td>
<td>414±5</td>
<td>7.1±0.6</td>
<td>115.6±3.6</td>
</tr>
<tr>
<td>A1AR-KO+ sildenafil</td>
<td>410±6</td>
<td>7.4±0.5</td>
<td>114.8±2.9</td>
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</tbody>
</table>

Values are means±SEM. HR—heart rate (beats/min); LVEDP—left ventricular end-diastolic pressure (mm Hg); LVDP—left ventricular developed pressure (mm Hg). \(^{a}p<0.05\) vs. A1AR-KO+ saline; \(^{b}p<0.05\) vs. A1AR-KO+sildenafil.
2.7. Statistics

All measurements are expressed as means ± SE. The data were analyzed by either unpaired \( t \)-test or one-way ANOVA. If a significant value of \( F \) was obtained in ANOVA, the Student–Newman–Keuls post hoc test was further used for pairwise comparisons. Paired \( t \)-test was used to compare any pair of pre- and post-treatment values for the same parameter. \( p < 0.05 \) was considered significant.

3. Results

3.1. Left ventricular cardiac function

There was no significant difference in the basal functional parameters (i.e., heart rate, LVDP, and LVEDP) between the groups after 20 min of stabilization (Table 1). Except in the A1AR-KO mice treated with saline, no significant changes in the rate pressure product were observed during post-ischemia (Fig. 2) between the groups. Post-ischemic coronary flow was also not significantly different among the groups as shown in Table 2.

3.2. Myocardial infarction

Following I/R, myocardial infarct size (mean ± SEM; % of risk area) was reduced in wild-type mice treated with sildenafil (5.6±0.9) versus saline group (27.3±2.1; \( p < 0.05 \); \( n=6/each \); Fig. 3). In contrast, sildenafil failed to reduce infarct size in the A1AR-KO mice (31.6±1.9 vs. 32.3±1.5 with saline, \( p > 0.05 \)).

Moreover, the selective A1AR antagonist DPCPX blocked the infarct-limiting effect of sildenafil in the wild-type mice (27.3±3.2, \( p < 0.05 \)). There was no significant difference in the infarct size between controls and animals treated with DPCPX or DMSO, the solvent for DPCPX.

3.3. Effect of sildenafil on cardiomyocyte necrosis

Following I/R, myocardial infarct size (mean ± SEM; % of risk area) was reduced in wild-type mice treated with sildenafil (5.6±0.9) versus saline group (27.3±2.1; \( p < 0.05 \); \( n=6/each \); Fig. 3). In contrast, sildenafil failed to reduce infarct size in the A1AR-KO mice (31.6±1.9 vs. 32.3±1.5 with saline, \( p > 0.05 \)).

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4. Discussion

The major goal of this study was to test the hypothesis whether A1AR plays an obligatory role in protection against I/R injury with sildenafil in the intact heart and cardiomyocytes. As previously shown in the ICR mice also [4], sildenafil caused an impressive reduction of infarct size in hearts isolated from C57BL-WT mice. The protection was abolished by both selective A1AR antagonist DPCPX as well as genetic deletion of A1AR. Similar results were obtained in an independent acute model of primary adult murine cardiomyocytes that were subjected to SI/RO in vitro. The number of trypan blue-positive necrotic cells following SI/RO was significantly lower in the sildenafil-treated group as compared with the saline-treated control cardiomyocytes. Also, sildenafil-induced protection against SI/RO was abolished by DPCPX in the wild-type cardiomyocytes or in cardiomyocytes isolated from A1AR-KO mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pre-ischemia (ml/min)</th>
<th>Reperfusion (ml/min)</th>
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<tbody>
<tr>
<td>WT + saline</td>
<td>2.1±0.4</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td>WT + sildenafil</td>
<td>2.2±0.3</td>
<td>2.4±0.3</td>
</tr>
<tr>
<td>WT + DPCPX + sildenafil</td>
<td>2.4±0.7</td>
<td>2.8±0.6</td>
</tr>
<tr>
<td>WT + DPCPX + saline</td>
<td>2.2±0.2</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>WT + DMSO + sildenafil</td>
<td>1.9±0.4</td>
<td>2.2±0.4</td>
</tr>
<tr>
<td>WT + DMSO + saline</td>
<td>2.5±0.3</td>
<td>2.7±0.2</td>
</tr>
<tr>
<td>A1AR-KO + saline</td>
<td>1.9±0.2</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>A1AR-KO + sildenafil</td>
<td>2.7±0.1</td>
<td>2.9±0.1</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of sildenafil, DPCPX, and genetic deletion of A1AR on ventricular functional recovery following global ischemia–reperfusion.
mice. Taken together, these combinatorial approaches of pharmacologic inhibition and gene knockout along with the use of in vitro and intact organ model provide conclusive evidence that activation of A1AR plays an essential role in sildenafil-induced protection in the heart.

Adenosine is a ubiquitous biological compound formed as a consequence of the breakdown of ATP during ischemic preconditioning. The adenosine receptor system is an intrinsic mechanism which has a role in modifying cell death and injury, inflammatory processes, and cardiac and vascular remodeling during/after ischemic or hypoxic insult and also protect the heart against the injury of I/R and inflammation (reviewed in [20]). It has been shown that inhibition of A1AR activation prevents protection with ischemic preconditioning in rabbits [9], dogs [21], and isolated human right atrial trabeculae [22]. Moreover, A1AR deletion prevents protection against myocardial infarction in vivo following ischemic preconditioning in mice [23]. Activation of A1ARs has been shown to protect the heart from I/R-induced stunning as well as infarction [24,25]. Moreover, cardiac-selective A1AR overexpression demonstrated enhanced ischemic tolerance as evidenced by the reduction of infarction and improved recovery of contractile function [26]. Conversely, A1AR-KO mice were shown to have diminished recovery of left ventricular function compared with WT controls [27].

Fig. 3. Top: Representative sections of the heart showing infarction (white) with 10% TTC staining. More viable tissue (red) is prominent in the sildenafil-treated WT mice as compared to the A1AR-KO mice and DPCPX-treated mice. Bottom: Effect of sildenafil on myocardial infarct size: role of A1AR. Isolated perfused hearts were subjected to 30 min global ischemia and 1 h reperfusion in Langendorff mode. Values are means±SE (n=6 heart samples per group). Group II (WT) and VIII (A1AR-KO) received sildenafil (0.71 mg/kg, i.p.). Group III received DPCPX (0.1 mg/kg) 30 min before sildenafil treatment. Group (I, VII) and (IV) served as controls for sildenafil and DPCPX, respectively. *p<0.05 vs. all other groups.
present study, we observed that treatment with sildenafil failed to reduce infarct size in the A1AR-KO as compared to the WT controls. These observations were further supported by the ability of DPCPX to block the infarct limiting effect of sildenafil in the WT control hearts. Similar results were obtained when these experiments were reproduced in adult cardiomyocytes subjected to simulated ischemia/reoxygenation. Schulte et al. [28] reported abrogation of the infarct sparing effect of remote (cerebral) ischemic preconditioning in A1AR-KO hearts while post-ischemic functional tolerance was not altered by A1AR deletion. It is well known that extracellular adenosine acts via four known G-protein-coupled receptor (GPCR) sub-types including A1, A2A, A2B, and A3 receptors [30]. Adenosine A1 and A3 receptors inhibit adenyl cyclase and stimulate phospholipase C via activation of pertussis toxin sensitive G proteins Gi and/or Go. A2A and A2B are positively coupled to adenyl cyclase but also activate alternate signaling pathways [31,32]. However, it is unlikely that the lack of protective effect of sildenafil in the A1AR-KO mice is due to compensatory changes in the expression of other receptor subtypes because A2A, A2B, or A3 receptors were not altered in A1AR-KO compared with WT hearts [27].

Our data raise the intriguing possibility that sildenafil may have caused enhanced expression of A1AR on cardiomyocytes, particularly 24 h after treatment. In support of this notion, recent studies have demonstrated that exogenously generated NO as well lipopolysaccharide (LPS) increased A1AR expression in pheochromocytoma 12 cells and in primary cultures of cortical neurons [29]. With LPS, the A1AR expression was dependent on the induction of inducible nitric oxide synthase (iNOS) because inhibition of NOS activity suppressed this response in these studies. It appears that sildenafil might have worked through NO-dependent increased A1AR expression. This is because sildenafil is known to cause a rapid induction of eNOS and iNOS mRNA and a significant increase in cardiac expression of the proteins within 24 h of treatment in the heart [4]. However, considering the shorter duration of action of DPCPX (approximately 5 h) in antagonizing the actions of adenosine [33], it is not clear how this compound would have been able to block the protective effect of upregulated A1AR 24 h later. It is possible that such an action of DPCPX may be indicative of a lack of specificity of action. Alternatively, sildenafil may have caused enhanced release of adenosine through generation of NO which would have activated the A1AR receptor and led to subsequent protection from I/R injury 24 h later. Additional studies are needed to address this hypothesis.

In summary, for the first time, we have shown that sildenafil induces delayed cardioprotective effects against myocardial I/R injury through selective activation of A1AR. Considering the similarity of action of other PDE-5 inhibitors including Vardenafil and Tadalafil, it is possible that these drugs may also require A1AR activation for protecting the heart against I/R. We therefore propose that the class of PDE-5 inhibitors including sildenafil could be a novel strategy to selectively activate A1AR function for the protection of the heart against myocardial infarction following I/R injury and remodeling.

Acknowledgments

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