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RESEARCH PAPER

Sildenafil reduces L-NAME-induced severe hypertension and worsening of myocardial ischaemia–reperfusion damage in the rat

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Background and purpose: Phosphodiesterase-5 inhibitors are beneficial in pulmonary hypertension and congestive heart failure, the two conditions associated with coronary heart disease and ischaemia. We investigated whether sildenafil counteracts the cardiovascular alterations induced by N^{ω}-nitro-L-arginine methyl ester (L-NAME) in the rat.
Experimental approach: Sildenafil was given orally to rats at doses of 0.37, 0.75 or 1.5 mg $kg^{-1}day^{-1}$ for four weeks, either alone or with L-NAME (35-40 mg kg^{-1} day^{-1} in the drinking water). Systolic blood pressure and urinary parameters (6-keto-prostaglandin $F_{1\alpha}$, thromboxane B_2, 8-isoprostane-prostaglandin $F_{2\alpha}$ and nitrite/nitrate) were measured in conscious rats. Isolated hearts were subjected to low flow ischaemia–reperfusion, and myocardial levels of guanosine 3′, 5′cyclic monophosphate (cGMP) were determined. Endothelial vascular dysfunction was examined in aortic rings.
Key results: Sildenafil dose-dependently prevented the rise in systolic blood pressure in L-NAME-treated rats. This activity was associated with a normalization of urinary 8-isoprostane-prostaglandin $F_{2\alpha}$ and other biochemical parameters. In perfused hearts, the post-ischaemic ventricular dysfunction was worse in preparations from L-NAME-treated rats than in controls. Sildenafil dose-dependently reduced this effect, and creatine kinase and lactate dehydrogenase release were lower too. cGMP levels, which were low in myocardial tissue from L-NAME-treated rats, were restored by sildenafil. In noradrenaline-precontracted aortic rings from L-NAME-treated rats acetylcholine lost its vasorelaxant effect, and sildenafil restored it.
Conclusion and implications: In a rat model of chronic nitric oxide deprivation, where hypertension and aggravation of post-ischaemic ventricular dysfunction are associated with loss of vascular endothelium-relaxant function, sildenafil provided significant cardiovascular protection, primarily by maintaining tissue cGMP levels.
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Keywords: L-NAME-induced hypertension; myocardial ischaemia–reperfusion; sildenafil

Abbreviations: AII, angiotensin II; AUC, area under the curve; cAMP, adenosine 3′,5′cyclic monophosphate; cGMP, guanosine3′,5′cyclic monophosphate; CK, creatine kinase; CPP, coronary perfusion pressure; E_{max}, maximal relaxant effect; ELISA, enzyme-linked immunosorbent assay; HR, heart rate; 8-iso-$PGF_{2\alpha}$, 8-isoprostane-prostaglandin $F_{2\alpha}$; KHS, Krebs Henseleit solution; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; LDH, lactate dehydrogenase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; LVP, left ventricular pressure; LVDevP, left ventricular-developed pressure; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; NO, nitric oxide; NOS, nitric oxide synthase; NOx, nitrite/nitrate; PDE-5, phosphodiesterase-5; SBP, systolic blood pressure; TXB_2, thromboxane B_2

Introduction

The discovery in 1989 of sildenafil, a selective inhibitor of phosphodiesterase-5 (PDE-5), was the result of extensive research on chemicals targeting PDE-5 that might be useful in the treatment of coronary heart disease (Moreland *et al*., 1998). However, soon after the clinical introduction of sildenafil for the treatment of erectile dysfunction, a number of reports of adverse cardiac events raised concerns about its safety in cardiovascular disorders (Feenstra *et al*., 1998; Kloner, 2000). It is well known that patients with erectile dysfunction share most of the risk factors of patients suffering from cardiovascular disease: hypertension and lipid abnormalities including low high-density lipoprotein and diabetes (Virag *et al*., 1985). Among men treated systematically with sildenafil, an incidence of serious cardiovascular

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events, listed as myocardial infarction, angina and coronary heart diseases, has been reported (Morales *et al*., 1998).

The pharmacodynamic and adverse event profile observed in clinical trials with sildenafil appears to be consistent with the *in vitro* profile of tissue distribution of PDE-5 and its mechanism of action as a selective inhibitor of PDE-5, including cross-talk between this enzyme isoform and PDE-3 (Vila-Petroff *et al*., 1999; Wallis *et al*., 1999). PDE-5 has been found in high concentrations in smooth muscles of the corpora cavernosa, and is expressed in a variety of other tissues such as the arterial vasculature, including pulmonary and coronary arteries, venous vasculature, skeletal muscles, visceral and tracheobronchial muscles and platelets (Senzaki *et al*., 2001; Gresser and Gleiter, 2002). However, the results of early studies with sildenafil reported by Morales *et al*. (1998) were not confirmed in random trials and retrospective analysis (Wysowski *et al*., 2002). Recent investigations have shown that the drug offers benefits in pulmonary arterial hypertension (Michelakis *et al*., 2002; Guazzi *et al*., 2004) and congestive heart failure (Bocchi *et al*., 2002), the two conditions associated with coronary heart disease. It has also been found that inhibition of PDE-5 with sildenafil attenuates cardiomyocyte apoptosis in a chronic model of doxorubicin cardiotoxicity (Fisher *et al*., 2005) and reduces the hypertrophic response to isoprenaline in rat heart, reducing myocardial leakage of creatine kinase (CK) and troponin T (Hassan and Ketat, 2005).

We therefore planned pharmacological experiments to investigate whether sildenafil counteracts the marked changes in the cardiovascular system induced by N^{ω}-nitro-L-arginine methyl ester (L-NAME) in the rat. Moreover, *ex vivo* heart preparations obtained from L-NAME-treated rats subjected to ischaemia–reperfusion were used to assess cardioprotective activity of sildenafil. On this matter, it is well known that L-NAME perfused directly through the isolated hearts markedly exacerbates postischaemic ventricular dysfunction upon reperfusion (Rossoni *et al*., 1995, 2000, 2004). Furthermore, even though the outcome of this study can be predicted from the well-known mechanism of action of PDE-5 inhibitors, the expected results will provide further support for the beneficial effects of sildenafil in cardiovascular diseases involving nitric oxide (NO) deficiency.

Methods

Animals

Male Wistar rats (Charles River Italia, Calco, LC, Italy) weighing 250–275 g were used. The animals were housed in a conditioned environment ($22\pm1^{\circ}$C, $55\pm5\%$ relative humidity, 12 h light/dark cycles) and were fed standard laboratory chow and water. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1996).

Experimental protocols

Experiments were conducted on 72 rats randomly divided into six groups of 12. The first group (control) received untreated drinking water and the second group received untreated drinking water plus 1.5 mg kg^{-1} sildenafil. The third group (L-NAME) received L-NAME (Sigma-Aldrich, Milan, Italy) added to its drinking water (1 mg ml^{-1}) for 4 weeks. At this concentration, the daily intake of L-NAME was approximately 35-40 mg kg^{-1} day^{-1}. The fourth, fifth and sixth groups received L-NAME-treated drinking water together with sildenafil at the dose of 0.37, 0.75 and 1.5 mg kg^{-1}, respectively. Sildenafil (Viagra 25, 50 or 100 mg tablets, Pfizer Limited, Sandwich, Kent, UK) was administered orally by gavage once a day for 4 weeks. The Viagra tablets were crushed and suspended in 0.5% carboxymethylcellulose (vehicle; 2 ml kg^{-1}). The largest dose of sildenafil used (1.5 mg kg^{-1}) is approximately equivalent to the clinical dose of 100 mg for a 70 kg patient. The rats were weighed, and their systolic blood pressure (SBP) and heart rate (HR) were measured weekly by the tail-cuff method. In metabolic cages, urine was collected to measure 24-h urinary excretion of protein, nitrite/nitrate (NOx) and prostaglandin metabolites such as 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$, a stable metabolite of prostacyclin), thromboxane B_2 (TXB_2, a stable metabolite of thromboxane A_2) and 8-isoprostane-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$). At the end of the 4-week treatment period, all rats were anaesthetized intraperitoneally with 60 mg kg^{-1} thiopentone sodium (Pentothal; Abbott, Campoverde, Latina, Italy) and the heart and thoracic aorta were immediately removed for *in vitro* studies or tissue determinations (see later).

Indirect SBP and HR measurements in conscious rats

Body weight and tail SBP were recorded weekly. SBP was measured by tail-cuff plethysmography (mod 8006; U Basile, Comerio, Varese, Italy) in unanaesthetized rats that had been placed in a warm cupboard (30°C) for 30 min. SBP values for individual rats were obtained from the average of three consecutive measurements and were considered valid only when these readings did not differ by more than 5 mm Hg. At the same time, HR was measured from the arterial pulse wave.

Urinary prostaglandin metabolites and NOx

Urinary 6-keto-$PGF_{1\alpha}$, TXB_2 and 8-iso-$PGF_{2\alpha}$ were assayed according to the general principles of the enzyme-linked immunosorbent assay (ELISA) technique (Porstmann and Kiessing, 1992) using specific and sensitive kits (Cayman Chemical, Ann Arbor, MI, USA). For 8-iso-$PGF_{2\alpha}$, samples were hydrolysed because more than 50% of total 8-iso-$PGF_{2\alpha}$ was present as lipoprotein esters. After 1 ml of 10 N NaOH was added to each 4 ml sample, the samples were warmed to 40°C for 1–2 h, then 5 ml of 2 N HCl was added to neutralize them. Samples were centrifuged at 600 *g* for 15 min, and the supernatants were collected for total 8-iso-$PGF_{2\alpha}$ immunoassay. The urinary protein concentration was measured by an improved Lowry assay (Lowry *et al*., 1951), and urinary NOx was quantitified with a colorimetric microplate assay kit (Cayman Chemical) that uses the Griess reagent.

Myocardial guanosine3′,5′cyclic monophosphate (cGMP) and adenosine 3′,5′cyclic monophosphate (cAMP)
cGMP and cAMP were measured in the rat hearts by specific ELISA kits according to the manufacturer's instructions (Cayman Chemical). The hearts ($n=5$ for group) were excised and immediately frozen in liquid nitrogen, then stored at −80°C until assayed. Frozen myocardial tissue samples in liquid nitrogen were ground to a fine powder in a stainless-steel mortar. Once the liquid nitrogen had evaporated, the frozen tissue was weighed and homogenized in 10 volumes of 0.1 M HCl to stop the action of PEs. Centrifugation was at 30 000 r.p.m. at room temperature and the supernatant was collected for quantitative immunoassay of cGMP and cAMP.

Rat isolated perfused heart experiments
Rat perfused heart preparations. Rat hearts ($n=7$ for group) were perfused as described previously (Rossoni *et al*., 1998). In brief, the heart was rapidly excised, and placed in cold (4°C) Krebs-Henseleit solution (KHS) with the following composition (in mM): NaCl 118, KCl 4.8, KH_2PO_4 1.2, $CaCl_2$ 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.5. The heart was mounted on the experimental set-up within 2 min after thoracotomy and perfused at 15 ml min^{-1} (Minipuls-3 peristaltic pump; Gilson, Villiers-Le Bel, France) through the aorta with KHS, maintained at 37°C and aerated with 95% O_2 + 5% CO_2 to stabilize normal pH, pO_2 and pCO_2. Coronary perfusion pressure (CPP) and left ventricular pressure (LVP) were measured with two HP-1280C pressure transducers (Hewlett-Packard, Waltham, MA, USA) connected to a Hewlett-Packard dynograph (HP-7754A). LVP was recorded with a polyethylene catheter, with a small latex balloon on the tip (no. 4; Hugo Sachs Elektronik, March-Hugstetten, Germany), inserted into the left ventricular cavity through the mitral valve opening. The volume of the balloon was adjusted to give peak left ventricular systolic pressure (LVSP) 90 ± 5 mm Hg with left ventricular end-diastolic pressure (LVEDP) 5–7 mm Hg. Hearts that could not achieve this level of contractile performance (8–10%) were excluded. Left ventricular developed pressure (LVDevP; peak LVSP−LVEDP) was also calculated. After a 15-min equilibration period, hearts were paced at 300 beats min^{-1} with an electrical stimulator (S-88; Grass Instruments, Quincy, MA, USA) using two silver electrodes attached to the right atrium, and a further 20 min of perfusion was carried out (pre-ischaemic period).

Effect of angiotensin II (AII) activity on CPP. To assess the integrity of endothelium-dependent relaxant mechanisms, at the beginning of each experiment the coronary vasculature reactivity to AII was evaluated in the same hearts subsequently subjected to ischaemia–reperfusion. AII (1 μg; Sigma-Aldrich) was injected as a bolus into the perfusion system.

Ischaemia–reperfusion in the perfused rat heart. Ischaemia was induced by reducing the flow rate from 15 to 1 ml min^{-1} for 20 min (ischaemic period). Normal flow rate (15 ml min^{-1}) was then restored and the perfusion was continued for another 30 min (reperfusion period). Throughout the experiment, a thermoregulated chamber held the heart temperature at 37°C to avoid hypothermia-induced cardioprotection. The total duration of each experiment did not exceed 90 min, during which time the experimental preparation remained stable.

CK and lactate dehydrogenase (LDH) activities in heart perfusates. The effluent obtained from the heart during the preischaemic and reperfusion periods was collected in an ice-cooled beaker as 2.5 min samples. Each sample was used for the determination of CK and LDH activities according to the method of Bergmeyer *et al*. (1970) and Hohorst (1963), respectively. The total activity was measured spectrophotometrically (Lambda-16; Perkin Elmer Italia, Monza, Milan, Italy) at 37°C using specific kits, according to the manufacturer's instructions (Sentinel Diagnostic, Milan, Italy).

Endothelial function in rat-isolated aortic rings
Segments of thoracic aorta from the different experimental groups of rats were cleaned of adherent connective tissue in KHS and cut into rings (3–5 mm long). The rings were carefully handled to avoid damage to the inner surface and suspended in organ bath chambers (10 ml) containing KHS gassed with a mixture of CO_2 (5%) and O_2 (95%) and maintained at 37°C (pH 7.4). Tissues were connected with silk sutures to force–displacement transducers (model 7004; U. Basile), and changes in isometric force were displayed on a Gemini chart recorder (model 7070; U. Basile). All rings were gradually stretched to a baseline resting tension of 1.5–1.7 *g*, which was maintained throughout the experiment, and the preparations were allowed to equilibrate for 60 min. To evaluate maximal contraction, the tissues were depolarized with 60 mM potassium chloride and washed with KHS. After 30 min, the rings were precontracted with noradrenaline (NA) (3×10^{-6} M), and, when the contractile response was stabilized (steady-state phase, 12–15 min), endothelial-dependent relaxation was evaluated by cumulative addition of acetylcholine (from 10^{-11} to 10^{-4} M). The direct relaxant effect of the NO-donor sodium nitroprusside (from 10^{-10} to 10^{-3} M) was also recorded.

Statistical analysis
Data are presented as mean ± s.e.m. The differences between the treatment groups were compared by the unpaired *t*-test or one-way analysis of variance, followed by the Student–Newman–Keuls *post hoc* test for multiple comparisons. $P<0.05$ was considered statistically significant. The area under the curve (AUC) was assessed using the computer programme Microcal Origin 3.5 (Microcal Software Inc., Northampton, MA, USA).

Results

Physical characteristics and urinary findings
Physical characteristics and urinary findings in control and L-NAME-treated rats are shown in Tables 1 and 2. At the end

Table 1 Body weight, water intake, urine volume and urinary protein at the end of a 4-week schedule of sildenafil in control and L-NAME rats

Group	Body weight (g)	Water intake (ml day^{-1} 100 g b.w.)	Urine volume (ml day^{-1} 100 g b.w.)	Urinary protein (mg day^{-1} 100 g b.w.)
Control	379.5±8.7	9.4±0.8	8.5±0.8	37.2±2.5
Sildenafil 1.5 mg kg^{-1}	380.1±5.9	10.2±0.9	9.0±0.6	37.3±2.3
L-NAME	337.8±9.6*	9.5±1.1	7.9±1.0	48.7±3.3*
L-NAME + sildenafil 0.37 mg kg^{-1}	357.2±9.3	10.8±1.5	9.3±0.5	43.2±4.9
L-NAME + sildenafil 0.75 mg kg^{-1}	371.8±7.4	11.0±1.4	9.8±1.2	40.9±4.2
L-NAME + sildenafil 1.5 mg kg^{-1}	382.4±4.7§	9.7±1.3	8.9±0.8	35.5±2.1§

Abbreviation: L-NAME, N^{ω}-nitro-L-arginine methyl ester.
L-NAME was given to rats in the drinking water at the concentration of 1 mg ml^{-1} (approximately 35–40 mg kg^{-1} day) for 4 weeks. Sildenafil, alone or in combination with L-NAME, was given orally once a day for 4 weeks. Data are means±s.e.m. ($n=12$ per group).
*$P<0.05$ versus control, $^{\S}P<0.05$ versus L-NAME.

Table 2 Urinary arachidonic acid metabolites and NOx at the end of a 4-week schedule of sildenafil in control and L-NAME rats

Group	6-keto-$PGF_{1\alpha}$ (ng day^{-1} 100 g b.w.)	TXB_2 (ng day^{-1} 100 g b.w.)	8-iso-$PGF_{2\alpha}$ (ng day^{-1} 100 g b.w.)	NOx (μmol day^{-1} 100 g b.w.)
Control	11.55±0.93	2.35±0.28	34.7±2.1	402.8±28.3
Sildenafil 1.5 mg kg^{-1}	11.04±0.75	1.42±0.17*	29.5±3.1	620.5±57.4**
L-NAME	14.78±1.02*	5.97±0.63***	49.8±1.8**	185.3±21.2***
L-NAME + sildenafil 0.37 mg kg^{-1}	12.53±0.54	4.85±0.39	44.5±2.3	238.3±25.6
L-NAME + sildenafil 0.75 mg kg^{-1}	11.75±1.06	3.94±0.56*§	40.1±1.6*§	297.5±46.7*§
L-NAME + sildenafil 1.5 mg kg^{-1}	10.82±1.03§	2.85±0.36$^{\#}$	32.8±2.0$^{\#}$	361.4±25.4$^{\#}$

Abbreviations: 8-iso-$PGF_{2\alpha}$, 8-isoprostane-prostaglandin $F_{2\alpha}$; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NOx, nitrite/nitrate; TXB_2, thromboxane B_2.
L-NAME was given to rats in the drinking water at a concentration of 1 mg ml^{-1} (approximately 35–40 mg kg^{-1} day) for 4 weeks. Sildenafil, alone or in combination with L-NAME, was given orally once a day for 4 weeks. Data are means±s.e.m. ($n=12$ per group). *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control; $^{\S}P<0.05$, $^{\#}P<0.01$ versus L-NAME.

of chronic inhibition of NO-synthase (NOS), all the animals appeared generally healthy, and there was no significant difference in water intake and urinary volume compared with control rats. Treated rats, however, showed 11% weight loss ($P<0.05$ versus control rats). Urinary proteins and 6-keto-$PGF_{1\alpha}$ were slightly raised ($P<0.05$), and the excretion of TXB_2 and 8-iso-$PGF_{2\alpha}$ was more pronounced ($P<0.001$ and <0.01, respectively) than in control animals. Urinary NOx was reduced by 54% ($P<0.001$ versus control rats). The 4-week oral schedule of sildenafil (1.5 mg kg^{-1}) had no effect on the health of control animals, which showed no differences in body weight, water intake, urinary volume and proteins compared with untreated control rats (Tables 1 and 2). However, this dose of sildenafil reduced the urinary levels of TXB_2 (40%; $P<0.05$) and increased NOx (54%; $P<0.01$) compared with untreated controls (Table 2). The concomitant administration of sildenafil to L-NAME rats prevented the lowering effect of L-NAME on body weight and urinary proteins. Furthermore, urinary levels of 6-keto-$PGF_{1\alpha}$, TXB_2, 8-iso-$PGF_{2\alpha}$ and NOx were restored in a dose-dependent manner to values comparable to those of control animals (Tables 1 and 2).

SBP and HR in conscious rats

Time-related changes in tail-cuff SBP and the final HR data are shown in Figure 1. In sildenafil-treated control rats there were no changes. In contrast, in L-NAME rats SBP rose progressively, reaching 183±6 mm Hg at the end of the 4-week treatment period compared with 129±8 mm Hg in control rats ($P<0.001$). The HR dropped slightly (21%; $P<0.05$) compared with controls (312±22 beats min^{-1}). The increase in SBP caused by chronic inhibition of NOS was antagonized by sildenafil, depending on the dose. The antihypertensive effect was particularly noticeable at the dose of 1.5 mg kg^{-1} as in this group of rats the SBP (134±5 mm Hg) was in the same range as in the control animals, and HR was normal (Figure 1).

Myocardial cGMP and cAMP

Figure 2 shows the myocardial levels of cGMP and cAMP with sildenafil in control and L-NAME rats. In heart tissues from control sildenafil-treated rats (1.5 mg kg^{-1}) cGMP (99±13 fmol mg^{-1} protein) and cAMP (70±10 pmol mg^{-1} protein) rose 2.4-fold ($P<0.001$) and 1.8-fold ($P<0.01$), respectively, over the corresponding control group. In myocardial preparations from L-NAME rats, however, cGMP and cAMP dropped 69% ($P<0.01$) and 57% ($P<0.05$), respectively (Figure 2). In heart tissues from L-NAME rats, sildenafil dose-dependently increased both cGMP and cAMP levels, and at the largest dose used (1.5 mg kg^{-1}) the drug restored ($P<0.05$) both cGMP and cAMP to levels above those found in myocardial tissues from untreated control rats, but not that level obtained in control rats treated with sildenafil alone.

Rat isolated perfused heart

AII activity on CPP. A bolus injection of 1 μg AII into the perfusion system of the hearts from control rats at the end of 4 weeks induced a prompt increase in CPP, which peaked at

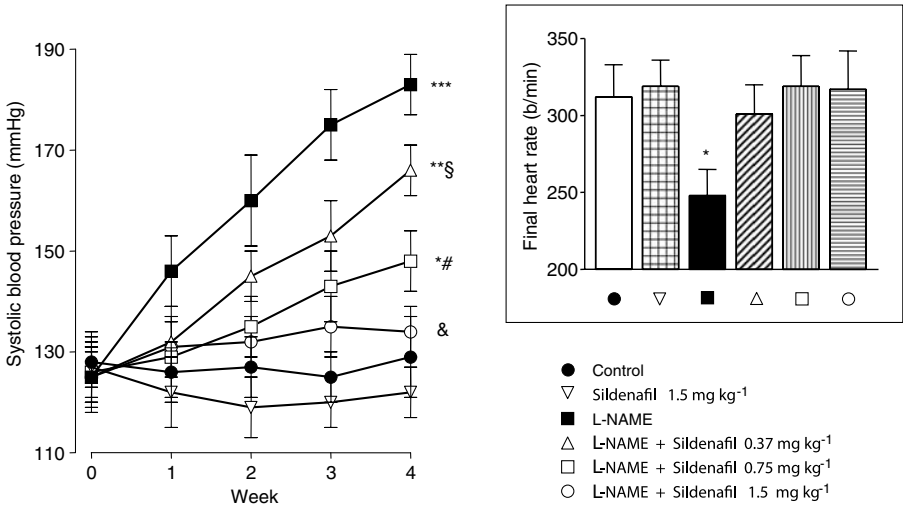

Figure 1 Time course of SBP and final HR (right panel) in control and L-NAME rats treated for 4 weeks with sildenafil alone or in combination with L-NAME. Data are means ± s.e.m. (vertical lines) of 12 animals per group. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus control; $^{\S}P<0.05$, $^{\#}P<0.01$ and $^{\&}P<0.001$ versus L-NAME alone.

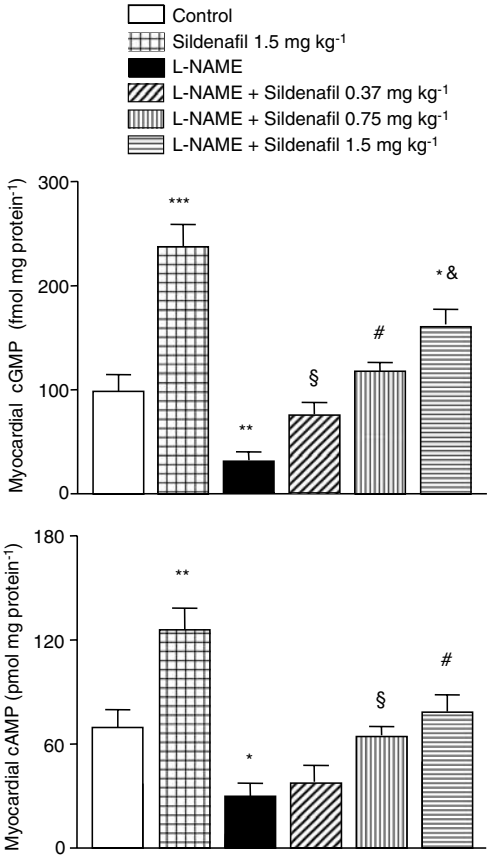

Figure 2 cGMP and cAMP in myocardial tissue from control and L-NAME rats treated for 4 weeks with sildenafil alone or in combination with L-NAME. Data are means ± s.e.m. (vertical lines) of five hearts per group. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus control; $^{\S}P<0.05$, $^{\#}P<0.01$ and $^{\&}P<0.001$ versus L-NAME alone.

20 ± 2 mm Hg above basal values (Figure 3). Sildenafil, 1.5 mg kg^{-1}, reduced the vasopressor effect of the autacoid by 46% ($P<0.05$ compared with controls). The AII-induced CPP increase in hearts from L-NAME rats was 2.7 times that in control hearts ($P<0.001$). This hyper-responsiveness of the coronary vasculature to AII clearly indicates a dysfunction of vascular endothelium-dependent relaxant function, which was antagonized in a dose-dependent manner in heart preparations from rats chronically treated with sildenafil. At the highest dose used, the drug almost abolished the hyper-responsiveness of the coronary vasculature to AII (Figure 3).

Ischaemia–reperfusion in the rat perfused heart. The time-courses of LVEDP and LVDevP in ischaemic–reperfused hearts from control and L-NAME rats at the end of 4 weeks are depicted in Figures 4 and 5. During the ischaemic period, the LVEDP of heart preparations from control animals began to rise progressively after a standstill (ventricular contracture), peaking at approximately 20 min (from 5 ± 1 to 30 ± 3 mm Hg; $P<0.001$). LVEDP then dropped slightly during reperfusion but at the end of this period was still significantly elevated (24 ± 2 mm Hg; $P<0.001$ versus pre-ischaemic values) (Figure 4). In these heart preparations, LVDevP was significantly depressed during reperfusion, and at the end of this period the myocardial contractility had recovered only 47% ($P<0.01$) of the preischaemic value (89 ± 6 mm Hg; Figure 5). In preparations from control animals, sildenafil (1.5 mg kg^{-1}) showed significant cardioprotection. The AUC of LVEDP and LVDevP were reduced 82% ($P<0.001$) and increased 2.1 times ($P<0.001$), respectively, compared with controls (Figures 4 and 5). The hearts from L-NAME rats subjected to ischaemia–reperfusion showed marked worsening of postischaemic ventricular dysfunction (Figures 4 and 5). At the end of the reperfusion, the LVEDP was significantly higher (57 ± 4 mm Hg) than in hearts from control rats (Figure 4). The LVDevP was further depressed (11 ± 5 mm Hg), with minimal recovery of myocardial contractility (Figure 5). In L-NAME rats sildenafil achieved a dose-dependent reduction of ischaemia–reperfusion damage, particularly significant with 1.5 mg kg^{-1}. The AUC for LVEDP and LVDevP demonstrated that the drug was cardioprotective (Figures 4 and 5). In line with the improvement of postischaemic ventricular dysfunction, sildenafil

dose-dependently restored the elevated CPP during reperfusion in preparations from both control and L-NAME rats (data not shown).

CK and LDH activity in heart perfusates. Figures 6 and 7 show the amount of CK and LDH activity in the coronary effluent collected during the preischaemic and reperfusion periods. There were no differences between the various groups in either CK or LDH released during the preischaemic period. However, during reperfusion, CK rose progressively in preparations from control and L-NAME rats, with peak increments of 9.2-fold ($P<0.001$) and 17.1-fold ($P<0.001$), respectively, over the corresponding values in the preischaemic period (Figure 6). The pattern was similar for LDH activity; this increased the perfusate of preparations from control and L-NAME rats by 9.8-fold ($P<0.001$) and 18.8-fold ($P<0.001$), respectively (Figure 7). In the hearts from control animals given sildenafil (1.5 mg kg^{-1}), the amount of both CK and LDH released during the reperfusion period was markedly reduced ($P<0.001$), almost to the preischaemic levels (Figures 6 and 7). In heart preparations from L-NAME rats, sildenafil dose-dependently prevented the release of CK and LDH during reperfusion. In this set of experiments, the inhibitory activity of sildenafil was particularly marked at the dose of 1.5 mg kg^{-1}. The AUC for CK and LDH were reduced by 86% ($P<0.001$) and 81% ($P<0.001$), respectively, compared with the corresponding values for control rats (Figures 6 and 7).

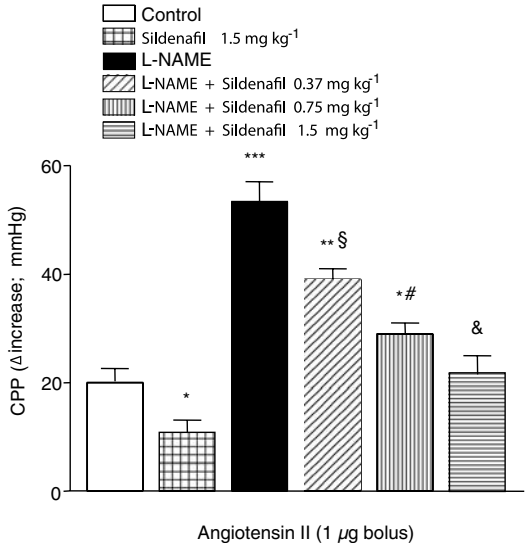

Figure 3 Changes in CPP induced by AII injected into rat perfused heart preparations during the preischaemic period. The hearts were obtained from control and L-NAME rats treated for 4 weeks with sildenafil alone or in combination with L-NAME. Data are means±s.e.m. (vertical lines) of seven hearts per group. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus control; $^{\S}P<0.05$, $^{\#}P<0.01$ and $^{\&}P<0.001$ versus L-NAME alone.

Endothelial function in rat isolated aortic rings

Figure 8 illustrates the endothelium-dependent relaxant effect of acetylcholine in NA-precontracted aortic rings. The contraction was of the same magnitude in preparations from control animals, whereas in rings from L-NAME rats the response to NA was 1.7 times ($P<0.05$) higher (data not shown). When NA-precontracted rings from control rats were exposed to cumulative concentrations of acetylcholine, marked vasorelaxation (maximal relaxant effect (E_{max}) 96±4%)) was obtained, whereas the response to acetylcholine in preparations from L-NAME rats was significantly lower (E_{max} 30±5%; $P<0.001$) (Figure 8). In contrast, when aortic rings from rats given L-NAME plus sildenafil were challenged with acetylcholine, the E_{max} of the neurotransmitter was restored, depending on the drug dose (Figure 8). The dose–response curves for NA-precontracted aortic rings with sodium nitroprusside were almost superimposable in preparations from control and L-NAME rats (data not shown).

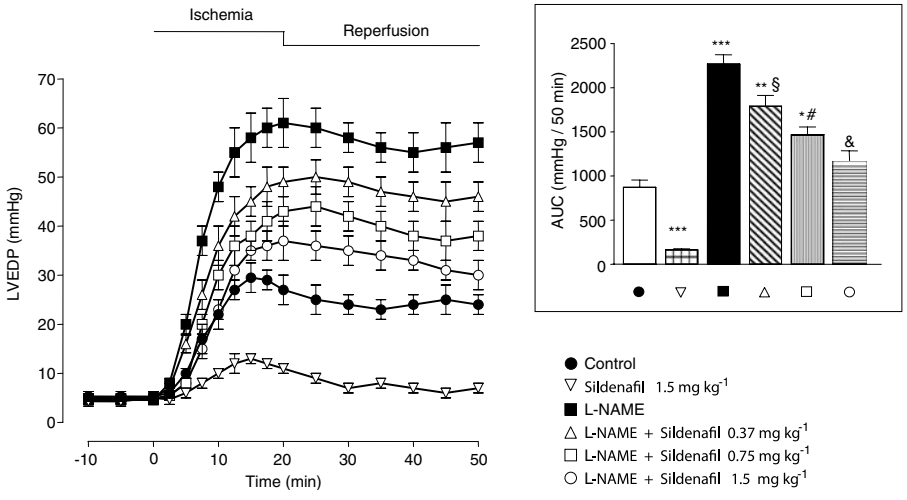

Figure 4 Time-course of LVEDP in rat perfused heart preparations subjected to ischaemia–reperfusion. The hearts were obtained from control and L-NAME rats treated for 4 weeks with sildenafil alone or in combination with L-NAME. Bar graph shows the AUC related to LVEDP curves (right panel). Data are means±s.e.m. (vertical lines) of seven hearts per group. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus control; $^{\S}P<0.05$, $^{\#}P<0.01$ and $^{\&}P<0.001$ versus L-NAME alone.

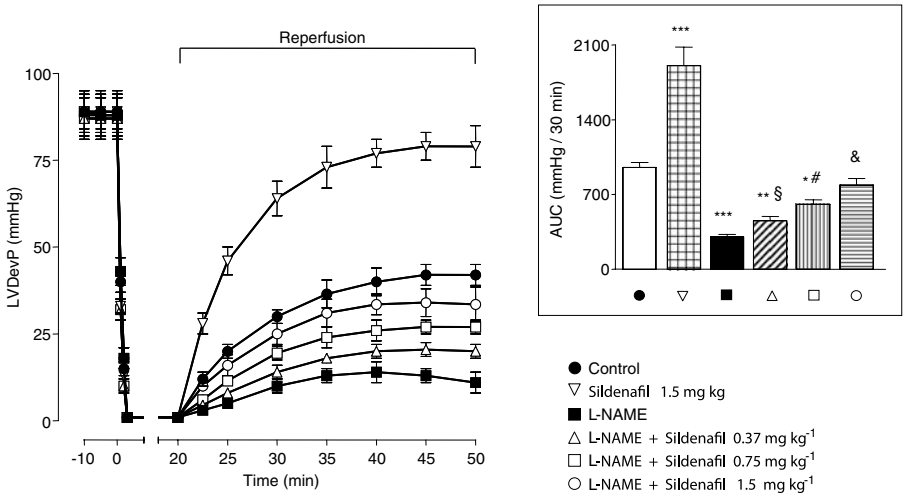

Figure 5 Time-course of LVDevP in rat perfused heart preparations subjected to ischaemia–reperfusion. The hearts were obtained from control and L-NAME rats treated for 4 weeks with sildenafil alone or in combination with L-NAME. Bar graph shows the AUC related to LVDevP curves (right panel). Data are means ± s.e.m. (vertical lines) of seven hearts per group. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus control; $^{\S}P<0.05$, $^{\#}P<0.01$ and $^{\&}P<0.001$ versus L-NAME alone.

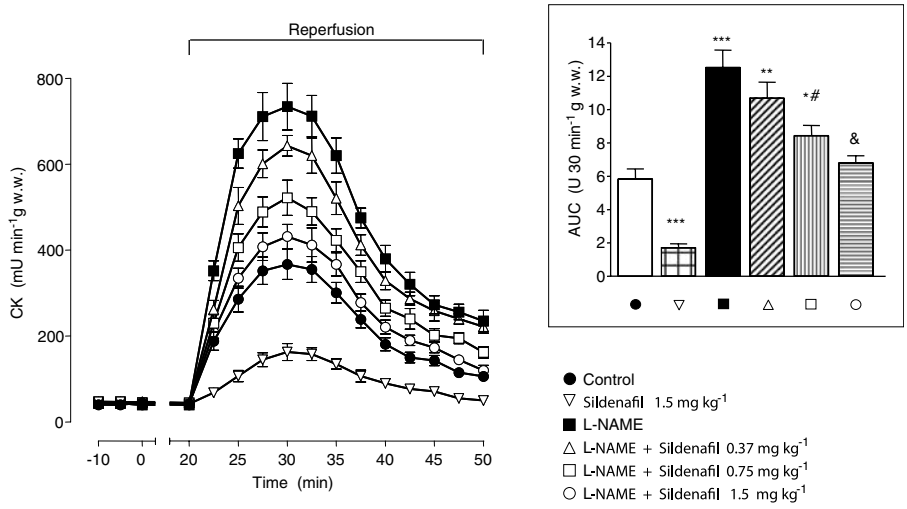

Figure 6 CK release profile in rat perfused heart preparations subjected to ischaemia–reperfusion. The hearts were obtained from control and L-NAME rats treated for 4 weeks with sildenafil alone or in combination with L-NAME. Bar graph shows the AUC related to CK curves (right panel). Data are means ± s.e.m. (vertical lines) of seven hearts per group. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus control; $^{\S}P<0.05$, $^{\#}P<0.01$ and $^{\&}P<0.001$ versus L-NAME alone.

Discussion

The present study provides evidence that the selective PDE-5 inhibitor, sildenafil, is effective at preventing and reversing the early consequences of vascular alterations in rats with NO deficiency induced by chronic inhibition of NOS by adding L-NAME to the drinking water. The mechanism(s) involved in the blood pressure (BP) elevation by L-NAME is well documented in the rat (Ribeiro *et al*., 1992; Pollock *et al*., 1993; Liu *et al*., 1998) where a vascular endothelial dysfunction, together with activation of the renin–angiotensin system and oxidative stress, appear to have a prominent role. In line with the previous findings (Tomida *et al*., 2003; Pechanova *et al*. 2004), the present results indicate that the progressive elevation of systemic BP owing to NO deprivation reflects a significant drop in the urinary levels of NOx and increases of both TXB_2 and 8-iso-$PGF_{2\alpha}$.

This latter arachidonic acid metabolite, a potent vasoconstrictor acting through TXA_2-receptor activation, has been suggested as a marker of oxidative stress (Takahashi *et al*., 1992). Several investigators have proposed that oxidative stress contributes to the generation or maintenance of hypertension through inactivation of NO, inhibiting its vasodilator and natriuretic actions, and through non-enzymatic generation of vasoconstrictor isoprostanes from arachidonic acid peroxidation (Griendling and Alexander, 1997; Ortiz *et al*., 2001). The hypertension caused by NOS inhibition is thus associated with increased oxidative stress, and chronic oral quercetin, a flavonoid with antioxidant properties, has been shown to have a protective effect in rats with hypertension from L-NAME (Duarte *et al*., 2002).

Urinary levels of 6-keto-$PGF_{1\alpha}$ only rose slightly, in line with the results of Tomida *et al*. (2003), indicating that in L-NAME-treated rats the levels of cyclooxygenase-2 mRNA

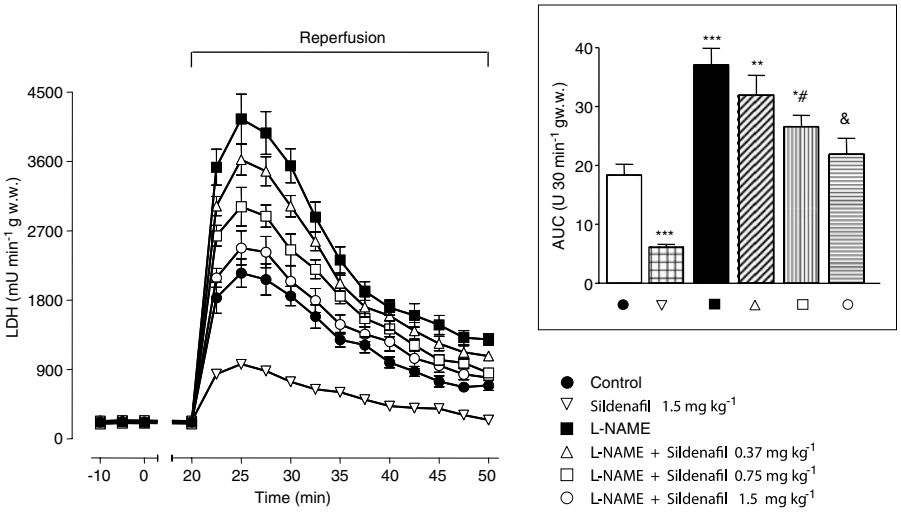

Figure 7 LDH release profile in rat perfused heart preparations subjected to ischaemia–reperfusion. The hearts were obtained from control and L-NAME rats treated for 4 weeks with sildenafil alone or in combination with L-NAME. Bar graph shows the AUC related to LDH curves (right panel). Data are means ± s.e.m. (vertical lines) of seven hearts per group. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus control; $^{\S}P<0.05$, $^{\#}P<0.01$ and $^{\&}P<0.001$ versus L-NAME alone.

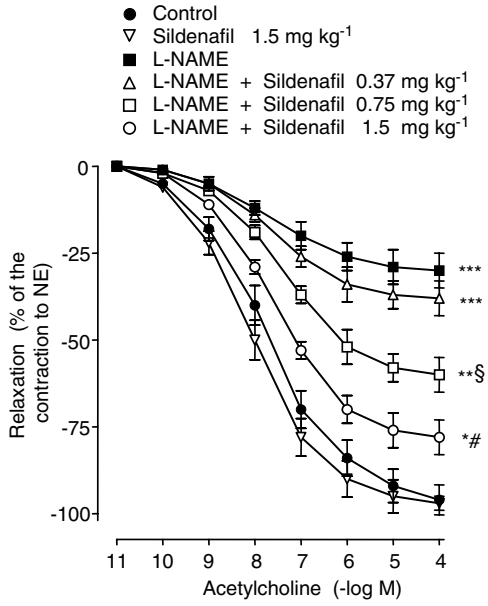

Figure 8 Cumulative concentration–response curves of acetylcholine in NA-precontracted aortic rings from control and L-NAME rats treated for 4 weeks with sildenafil alone or in combination with L-NAME. Data are means ± s.e.m. (vertical lines) of 12 preparations per group. Statistical differences at the maximum relaxant effect: *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus control; $^{\S}P<0.05$ and $^{\#}P<0.01$ versus L-NAME alone.

and protein were elevated in the kidneys and the thoracic aorta, suggesting that an increase in cyclooxygenase-2 expression might have a hypertensive effect partly associated with 8-iso-$PGF_{2\alpha}$ formation in L-NAME rats. In this combination study with L-NAME, sildenafil dose-dependently prevented the rise in BP, with restoration of urinary levels of NOx, TXB_2, 6-keto-$PGF_{1\alpha}$ and 8-iso-$PGF_{2\alpha}$. The beneficial effect of this drug in this model of hypertension might be primarily explained by an increase in accumulation of cGMP in the vasculature, where a certain degree of endothelial-dependent relaxant dysfunction was also observed. In fact, while acetylcholine loses its relaxant activity in NA-precontracted aortic rings from L-NAME rats, it regains its activity in preparations from animals given L-NAME plus sildenafil. In addition, the hyper-responsiveness of the perfused coronary artery to AII in heart preparations from L-NAME rats, prevented by concomitant administration of sildenafil, reinforces the theory that the vascular endothelium dysfunction extends to central and peripheral vessels. The present results are in line with the recent findings of Teixeira *et al*. (2006) showing that sildenafil and tadalafil increased cGMP and potently relax rat aortic rings through NO-dependent mechanisms. In line with our data, Ferreira-Melo *et al*. (2006) showed that sildenafil reduces cardiovascular remodelling associated with hypertensive cardiomyopathy in NOS inhibitor-treated rats. These authors stress that the beneficial effect of sildenafil was probably mediated by an increase in cardiac and vascular cGMP levels as reflected in circulating plasma cGMP levels.

Interesting results were recorded when the hearts from rats treated with L-NAME alone or in combination with sildenafil were perfused at a constant coronary flow and then subjected to ischaemia–reperfusion. The model of acute myocardial ischaemia adopted, useful for assessing intrinsic changes in cardiac performance, has certain limitations compared with true ischaemia (Henry *et al*., 1977). The control of coronary flow and ventricular volume may generate alterations of coronary and ventricular dynamics that could be amplified by L-NAME in view of its vascular effects. For this, a question arises whether the perfusion of the hearts at constant pressure rather than with constant flow could be a more convenient approach. Even though, the present data indicate that the chronic inhibition of NOS caused a severe exacerbation of postischaemic ventricular dysfunction as compared with control animals. This effect

was consistent with a further increase in LVEDP during ischaemia and a strong depression of LVDevP at reperfusion. As expected, these cardio-mechanical alterations involved significant increases in both CK and LDH activities at reperfusion. The exacerbation of ischaemia–reperfusion damage with L-NAME has already been seen and discussed in isolated rabbit hearts where the NO donors were highly protective (Rossoni *et al*., 1995, 2000, 2004).

The present experiments prove that selective inhibition of PDE-5 with sildenafil also dose-dependently prevents the L-NAME-induced enhancement of ischaemia–reperfusion damage. The protective activity of the drug might be explained by increased accumulation of cGMP in myocardial tissues, where the cyclic nucleotide may have been hydrolysed, PDE-5 enzymes having been recently demonstrated in the myocardium too (Giordano *et al*., 2001). Sildenafil dose-dependently raised cGMP levels in cardiac tissue of control and L-NAME rats. Evidence suggesting that the accumulated cGMP may have inhibited the PDE-3 enzymes responsible for cAMP degradation may explain the concomitant increase in cAMP (Vila-Petroff *et al*., 1999). However, the present findings contrast with those of Du Toit *et al*. (2005), indicating that the protective properties of sildenafil at a low concentration are due to cGMP-elevating and cAMP-suppressing effects in the ischaemic hearts of normal rats. According to these authors, the increase in cAMP in the cardiac tissues contributes to ischaemic Ca^{2+} overload and more severe ischaemic–reperfusion injury. The differences from the present findings are methodological, with important differences in L-NAME and sildenafil administration. However, in the light of the powerful anti-ischaemic activity of sildenafil in controls and in hearts with chronic NO deprivation, it is reasonable to assume that the changes in myocardial cAMP levels may have no biochemical significance, with no influence on cytosolic Ca^{2+} overload and worsening of ischaemic damage. According to Henry *et al*. (1977), the accumulation of Ca^{2+} in the mitochondrial fraction of cardiac myocytes and the increase in undissociated cross-bridges (actin–adenosine dinucleotide phosphate–myosin complex) are responsible for the cardio-mechanical changes, such as incomplete or delayed myocardial relaxation and ventricular contraction typical of the ischaemia–reperfusion model used in this study. Therefore, it is tempting to speculate that the accumulation of cGMP in cardiomyocytes owing to selective inhibition of PDE-5 by sildenafil may have restricted the depletion of energy stores in ischaemic cells, promoting dissociation of cross-bridges and reducing ventricular stiffness. In this respect, it has been shown that agents that increase intracellular cGMP may have a profound effect on the cytoplasmatic Ca^{2+} concentration, thereby mediating relaxation (Kai *et al*., 1987). It is conceivable that the Ca^{2+} transport system that removes Ca^{2+} from cytoplasm is one of the targets of cGMP-dependent protein kinase (Mery *et al*., 1991). Mechanism(s) suggested for sildenafil-induced cardioprotection involve increased induction of NOS (Salloum *et al*., 2003) or opening of the mitochondrial adenosine triphosphate-sensitive K^+ channels in the rabbit owing to modest vasodilatation caused by cGMP accumulation (Ockaili *et al*., 2002). This mild vasodilator effect is supposed to release agents such as adenosine, bradykinin or NO, which may trigger a preconditioning-like effect in the heart.

In conclusion, the present results indicate that, in a rat model of chronic NO deprivation where hypertension and exacerbation of myocardial postischaemic ventricular dysfunction cause a loss of vascular endothelial function, sildenafil, through a mechanism primarily based on accumulation of cGMP, offers significant vascular and cardiac protection.

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Conflict of interest

The authors state no conflict of interest.

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