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Sildenafil citrate concentrations not affecting oxidative phosphorylation depress H_2O_2 generation by rat heart mitochondria

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Abstract Sildenafil citrate (Viagra) is a potent and specific inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5), which exhibits cardioprotective action against ischemia/reperfusion injury in intact and isolated heart. The mechanism of its cardioprotective action is not completely understood, but some results suggested that sildenafil exerts cardioprotection through the opening of mitochondrial ATP-sensitive K^+ channels (mitoK_{ATP}). However, the impact of sildenafil citrate per se on isolated heart mitochondrial function is unknown. The goal of this study was to investigate the influence of the compound on mitochondrial function (bioenergetics, Ca^{2+}-induced mitochondrial permeability transition, and hydrogen peroxide (H_2O_2) generation) in an attempt to correlate its known actions with effects on heart mitochondria. It was observed that sildenafil citrate concentrations of up to 50 μM did not significantly affect glutamate/malate-supported respiration in states 2, 3, 4, oligomycin-inhibited state 3, and uncoupled respiration. The respiratory control ratio (RCR), the ADP to oxygen ratio (ADP/O), the transmembrane potential ($\Delta\Psi$), the phosphorylation rate, and the membrane permeability to H^+, K^+ and Ca^{2+} were not affected either. However, sildenafil citrate decreased H_2O_2 generation by mitochondria respiring glutamate/malate, and also decreased the formation of superoxide radical ($O_2^{\bullet -}$) generated in a hypoxantine/xantine oxidase system. It was concluded that sildenafil citrate concentrations of up to 50 μM do not affect either rat heart mitochondrial bioenergetics or Ca^{2+}-induced mitochondrial permeability transition, but it depresses H_2O_2 generation by acting as a superoxide dismutase (SOD)-mimetic. By preventing reactive oxygen species (ROS) generation, sildenafil citrate may preserve heart mitochondrial function.

Keywords Antioxidants · Heart · Ischemia · Mitochondrial bioenergetics · Oxidative stress · Permeability transition pore · Reperfusion · Sildenafil citrate · Viagra

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Introduction

In less than 20 years, sildenafil (1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulphonyl]-4-methylpiperazine) (Fig. 1), a potent and specific inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5), has evolved from a potential anti-angina drug into an on-demand oral treatment for erectile dysfunction (Viagra), and more recently, into a new orally active treatment for pulmonary arterial hypertension (Revatio) [1]. The action mechanism of sildenafil citrate is not completely understood, but it is believed that, by inhibiting the breakdown of nitric oxide

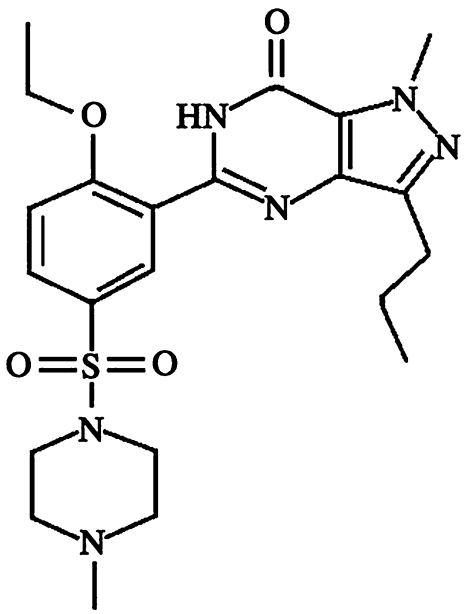


Fig. 1 Molecular structure of sildenafil

(NO)-driven cGMP, sildenafil citrate prolongs the relaxation and vasodilatation actions of cGMP on vascular/corpus cavernosal smooth muscle cells [1–3]. However, more recent studies have shown that sildenafil citrate also inhibits superoxide formation ($O_2^{\bullet -}$) by NADPH oxidases, suggesting that the therapeutic action of the compound may be mediated partly through antioxidant mechanisms [4–6].

Several studies have shown that sildenafil citrate exhibits a cardioprotective effect against ischemia/reperfusion injury in intact and isolated hearts [3, 7–11]. There is substantial evidence that the vasodilatating properties of sildenafil is the major factor underlying the cardioprotection, as vasodilatation would lead to release of endogenous mediators from endothelial cells (such as adenosine and bradykinin) leading to phosphorylation of endothelial NO synthases (NOS) and release of NO. This ultimately leads (via an increase in cGMP and activation of protein kinase G (PKG)) to activation of the mitochondrial ATP-sensitive K^+ channels (mitoK_{ATP}).

It is widely acknowledged that ischemia/reperfusion lead to mitochondrial, as well as cellular damage in cardiac cells. Furthermore, the phenomenological description of the mitochondrial changes that occur in ischemia/reperfusion injury, together with pharmacological studies on agents that protect against such changes, suggest that mitochondrial dysfunction might be important as a major causative agent in tissue injury and, also, the main target for intervention to limit the damage [12–15]. However, the precise mechanism by which sildenafil citrate exerts cardioprotection related with its action on mitochondrial function has not been analysed. Mitochondria, whose main function is ATP synthesis by oxidative phosphorylation, are also the most important cellular source of reactive oxygen species (ROS), the main targets for ROS regulatory and toxic actions, and the sources of signaling molecules that command cell cycle, proliferation, and apoptosis [16, 17]. Mitochondria regulate the life and cell death of cells by manipulating several factors, including bioenergetics, mitochondrial permeability transition pore, and mitochondrial redox-status [12].

The goal of this study was to investigate the effect of sildenafil citrate on rat heart mitochondrial function (bioenergetics, Ca^{2+}-induced mitochondrial permeability transition pore, and hydrogen peroxide (H_2O_2) generation), in an attempt to correlate the known actions of the compound with its effects on heart mitochondria. Considering the interest in elucidating the mechanism of action underlying the cardioprotective action of sildenafil citrate, the results obtained in this study will be a good starting point to investigate its potential protective effect on mitochondrial dysfunction induced by ischemia/reperfusion injury. Sildenafil citrate concentrations used in this study are within the concentration range used by other investigators in protection against necrosis and apoptosis in cardiomyocytes [18].

Materials and methods

Animals

Male Wistar rats (250–350 g), housed at 22 ± 2°C under artificial light for 12-h light/dark cycle and with access to water and food ad libitum, were used throughout the experiments. The experiments reported here were carried out in accordance with the National Requirements for Vertebrate Animal Research and in accordance with the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

Isolation of heart mitochondria

Heart mitochondria were prepared from male Wistar rats (6 weeks) by differential centrifugation according to conventional methods [19] with slight modifications. Briefly, the hearts were finely minced in an ice-cold isolation medium containing 250 mM sucrose, 1 mM EGTA, 10 mM Tris–HCl (pH 7.4), and 0.1% defatted bovine serum albumin (BSA). The minced tissue was suspended in 20 ml of isolation medium and homogenized with a tightly fitted homogenizer (Teflon/glass). Then, 0.5 mg protease type VIII (Sigma, P5390) per g of tissue plus 20 ml of

isolation medium was added to the homogenate. The suspension was incubated for 1 min at 4°C and then re-homogenized. The homogenate was centrifuged at 9,500*g* for 10 min. The supernatant was decanted and the pellet was gently re-suspended, in isolation medium, to its original volume. The suspension was centrifuged at 900*g* for 10 min, and the resulting supernatant was centrifuged at 9,500*g* for 10 min. The pellet was resuspended in 40 ml of washing medium (250 mM sucrose, 10 mM Tris–HCl, pH 7.2), and re-pelleted twice at 9,500*g* for 10 min. Finally, the pellet was resuspended in 0.5 ml of washing medium (at a protein concentration of about 17 mg ml^{-1}) and stored in ice. Heart mitochondrial preparations isolated by this procedure correspond to heterogeneous populations of subsarcolemmal and interfibrillar mitochondria. Protein content was determined by the biuret method [20], using BSA as a standard.

Measurement of respiratory activities

Oxygen consumption was polarographically monitored with a Clark-type electrode, in a closed glass chamber equipped with magnetic stirring, thermostated at 30°C. Mitochondrial protein (0.5 mg) was incubated in 1 ml of medium containing 130 mM sucrose, 5 mM Hepes (pH 7.2), 50 mM KCl, 2.5 mM K_2HPO_4, 2.5 mM $MgCl_2$, 0.1 mM EGTA in the absence and presence of sildenafil citrate for 3 min. At the end of the incubation time, mitochondrial protein was energised by adding 10 mM glutamate/5 mM malate. In order to induce state 3 respiration, adenosine diphosphate (ADP, 200 μM) was added. Inhibition of state 3 respiration was induced by adding 1 μg/ml oligomycin. Uncoupled respiration was initiated by the addition of 1 μM p-trifluoromethoxyphenylhydrazone (FCCP). O_2 consumption was calculated taking the saturation oxygen concentration to be 240 nmol O_2 ml^{-1}. The respiratory control ratio (RCR) and ADP to oxygen ratio (ADP/O) were calculated in accordance with a previously described method [21].

Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta\psi$) was measured in an open glass chamber equipped with magnetic stirring, thermostated at 30°C, using a lipophilic cation tetraphenylphosphonium (TPP^+)-selective electrode, as previously described [22]. To 1 ml of medium containing 130 mM sucrose, 5 mM Hepes (pH 7.2), 50 mM KCl, 2.5 mM K_2HPO_4, 2.5 mM $MgCl_2$, 0.1 mM EGTA, supplemented with 3 μM TPP^+ and 10 mM glutamate/5 mM malate, in the absence and presence of sildenafil citrate, 0.5 mg mitochondrial protein was added. In order to induce phosphorylation, adenosine diphosphate (ADP, 200 μM) was added. No correction was made for the "passive" binding of TPP^+ to the mitochondria membranes because the purpose of the experiments was to show relative changes in potential rather than absolute values. As a consequence, we can anticipate some overestimation for the $\Delta\psi$ values. Sildenafil citrate did not affect TPP^+ binding to mitochondria membranes or the electrode response.

Mitochondrial swelling

Mitochondrial osmotic volume changes were measured by the apparent absorbancy changes at 540 nm with a suitable spectrophotometer-recorder set up. Mitochondrial swelling methods were used to detect H^+ and K^+ mitochondrial inner membrane permeabilisation [23]. The reactions were carried out at 30°C, with 0.5 mg mitochondrial protein suspended in 2.5 ml of the required media, as described below.

Mitochondrial inner membrane permeabilisation to H^+ by sildenafil citrate was detected in K-acetate medium (135 mM K-acetate, 5 mM Hepes (pH 7.1), 0.1 mM EGTA, and 0.2 mM EDTA) supplemented with 2 μM rotenone. All assays were performed in the presence of 1 μg/ml valinomycin to permeabilise to K^+. A control assay was performed in the presence of 1 μM FCCP for total permeabilisation to H^+.

In order to detect K^+ mitochondrial inner membrane permeabilisation by sildenafil citrate, we used K-nitrate medium (135 mM KNO_3, 5 mM Hepes (pH 7.1), 0.1 mM EGTA, and 0.2 mM EDTA) supplemented with 2 μM rotenone. Control assay was performed in the presence of 1 μg/ml valinomycin for total permeabilisation to K^+.

Ca^{2+}-induced mitochondrial membrane transition pore (MPT)

In order to detect Ca^{2+}-induced MPT, we used a TPP^+ electrode and a medium containing 200 mM sucrose, 10 mM Mops–Tris (pH 7.4), 5 mM KH_2PO_4, 10 μM EGTA, supplemented with 2 μM rotenone, in the absence and presence of sildenafil citrate. Mitochondrial protein (0.5 mg), incubated in 1 ml of medium at 30°C for 3 min, was energised with 10 mM succinate, and one addition of Ca^{2+} (300 nmol Ca^{2+}) was used to induce MPT. Control assays, in both absence and presence of Ca^{2+} plus 1 μM cyclosporin A (CsA), were also performed.

Hydrogen peroxide generation

Hydrogen peroxide (H_2O_2) generation was measured fluorimetrically using a modification of the method previously described [24]. Briefly, mitochondrial protein (0.2 mg) was incubated at 30°C in 1.5 ml of medium containing 5 mM KH_2PO_4, pH 7.4, 0.1 mM EGTA, 145 mM KCl, 30 mM Hepes, 0.1 mM homovanillic acid, and 6 U/ml horseradish peroxidase. The reactions were started by adding 10 mM glutamate/5 mM malate and stopped 15 min later with 0.5 ml of cold 0.1 M glycine–NaOH (pH 12) containing 25 mM EDTA. The mitochondrial suspensions were centrifuged at 850*g* for 10 min. The fluorescence of supernatants was measured at 312 nm as excitation and 420 nm as emission wavelengths. The peroxide generation was calculated using a standard curve of H_2O_2.

Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) activity was evaluated using a spectrophotometrical assay, as described elsewhere [25]. Detection of SOD activity in this procedure is based on its ability to inhibit the reduction rate of nitroblue tetrazolium (NBT) by the superoxide radical ($O_2^{\bullet -}$) generated in a hypoxantine/xantine oxidase system. The assays were performed in 3 ml of reaction medium containing 50 mM KH_2PO_4 (pH 7.8), 1 mM EDTA, 100 μM NBT and 100 μM hypoxanthine. The reaction was initiated by the addition of xanthine oxidase (usually 0.025 U/ml) to produce a NBT reduction rate of 0.025 absorbancy units per min at 550 nm and 25°C. Sildenafil citrate (10, 20, 30, and 50 μM) was added to the incubation mixture and the reaction allowed continuing for 3 min at 25°C, with continuous magnetic stirring. The measurements were performed at 550 nm in a Jasco V560 UV/VIS Spectrophotometer, against a blank prepared in the absence of xantine oxidase. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50%. The SOD-like activity of sildenafil citrate was calculated using a standard curve prepared with SOD concentrations.

Statistical analysis

All the experiments were performed using three independent experiments with different mitochondrial preparations. The values are expressed as mean ± SE. Means were compared using one-way ANOVA test for multiple comparisons, followed by the Tukey's test. Statistical significance was set at $P < 0.05$.

Chemicals

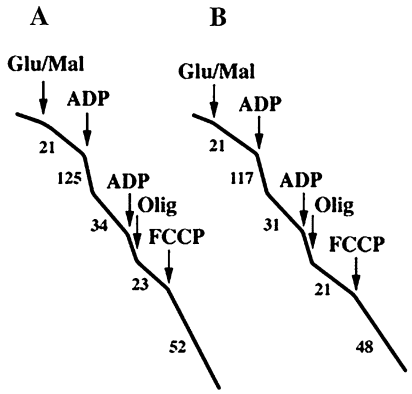
All chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA) except sildenafil citrate which was kindly supplied by Sandwich Laboratories, Pfizer Limited, Sandwich, Kent CT13 9NJ, UK.

Results

Effect of sildenafil citrate on rat heart mitochondrial bioenergetics

The effects of sildenafil citrate on glutamate/malate-supported respiratory rates (states 2, 3, 4, oligomycin-inhibited state 3 (state Olig), and FCCP-uncoupled respiration (state FCCP)) and respiratory indices RCR and ADP/O ratio of rat heart mitochondria are almost non-existent and insignificant at concentrations up to 50 μM (Fig. 2), suggesting that the compound had no significant effect on the respiratory capacity or phosphorylation efficiency of mitochondria. These results are confirmed by Fig. 3, where sildenafil citrate (50 μM) did not affect either the $\Delta\Psi$ induced by glutamate/malate-dependent respiration or the phosphorylation efficiency.

The lack of significant interference of sildenafil citrate with heart mitochondrial bioenergetics proves no effect on the respiratory electron chain and agrees with no permeabilisation of mitochondrial inner membrane. Our results confirmed its inability to affect mitochondrial inner membrane permeabilisation to H^+ and K^+, which were evaluated by the swelling of non-respiring mitochondria suspended in potassium acetate and potassium nitrate media, respectively (Fig. 4). Protonated acetate can cross the mitochondrial inner membrane, and then in the mitochondrial matrix, dissociate to the acetate anion and H^+, producing a proton gradient. A valinomycin-dependent swelling only occurs if the proton gradient is dissipated. Sildenafil citrate concentrations of up to 50 μM did not affect the rate of valinomycin-dependent mitochondrial swelling, indicating a lack of action on proton conductance through the mitochondrial inner membrane (Fig. 4a). Optimal swelling in potassium nitrate medium (KNO_3) is observed in conditions of K^+ permeabilisation, since the mitochondrial inner membrane is permeable to nitrate (NO_3^-). Maximal rate of swelling is observed by adding valinomycin to provide K^+ entry. Sildenafil citrate concentrations of up to 50 μM added, instead of valinomycin, did not induce swelling, indicating that the K^+ conductance through the inner mitochondrial membrane was not affected by the highest tested concentration of sildenafil citrate (Fig. 4b).



Sil (μM)	State 2	State 3	State 4	State Olig	State FCCP	RCR	ADP/O
0	27.9 ± 1.4	112.8 ± 8.7	42.7 ± 3.8	13.7 ± 1.4	48.3 ± 3.1	4.2 ± 0.3	2.4 ± 0.10
10	26.3 ± 0.5	106.0 ± 9.1	41.1 ± 4.2	13.5 ± 1.7	44.1 ± 5.2	4.3 ± 0.2	2.5 ± 0.05
20	25.0 ± 1.4	102.4 ± 9.8	40.5 ± 4.7	13.5 ± 0.9	40.4 ± 5.1	4.1 ± 0.3	2.5 ± 0.09
30	23.3 ± 1.3	95.7 ± 7.0	40.3 ± 3.3	12.9 ± 0.9	37.7 ± 4.3	3.7 ± 0.5	2.5 ± 0.10
50	25.7 ± 0.9	92.9 ± 5.2	43.7 ± 3.5	12.9 ± 1.2	40.5 ± 1.9	3.4 ± 0.6	2.4 ± 0.13

Fig. 2 Effect of sildenafil citrate on the respiratory parameters of rat heart mitochondria using glutamate/malate as a substrate. Freshly isolated mitochondria were incubated at 0.5 mg/ml under standard conditions, as described in the "Materials and methods" section. Reactions were started by adding glutamate/malate. The traces are typical recordings representative of three experiments obtained from different mitochondrial preparations. Trace **a**, mitochondria in the absence of sildenafil (Sil); Trace **b**, mitochondria in the presence of 50 μM Sil. Where indicated by arrows, 10 mM glutamate/5 mM malate (Glu/Mal); 200 μM ADP; 1 μg/ml oligomycin (Olig), and 0.5 μM FCCP, were added. Numbers on the records and table underneath are values of activities for state 2, state 3, state 4, state Olig, and state FCCP expressed in nmol O_2 mg^{-1} protein min^{-1}. The table underneath shows the mean ± SE of respiratory parameters at the different indicated concentrations of Sil

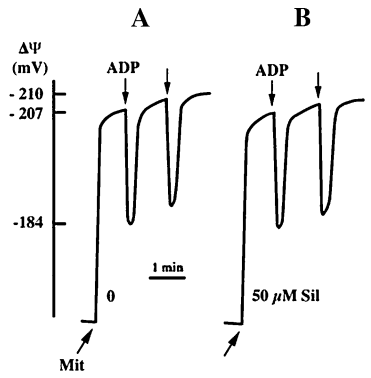
Effect of sildenafil citrate on Ca^{2+}-induced MPT of rat heart mitochondria

The effects of sildenafil citrate on the mitochondrial permeability transition (MPT) were evaluated by measuring mitochondrial transmembrane potential ($\Delta\psi$) drop, which is a typical phenomenon that follows the induction of MPT. In control conditions, the addition of 10 mM succinate produced $\Delta\psi$ of ≈ -220 mV (Fig. 5). Addition of a pulse of 300 nmol Ca^{2+}/0.5 mg protein led to a rapid depolarisation (decrease of $\Delta\psi$) followed by a partial repolarisation (recover of $\Delta\psi$) with subsequent total depolarisation of mitochondria. Mitochondria can tolerate a certain amount of Ca^{2+}, but ultimately their capacity of Ca^{2+}-loading is overwhelmed and mitochondria completely depolarise due to a change in the inner membrane permeability. This is prevented in the presence of 1 μM cyclosporine A (CsA), a specific inhibitor of MPT [26]. Incubation of mitochondria with sildenafil citrate concentrations of up to 50 μM, for 3 min before energisation with succinate, did not affect the capacity of mitochondria to accumulate Ca^{2+}, suggesting that this compound has no ability to affect Ca^{2+}-induced MPT.

Effect of sildenafil citrate on rat heart oxidative stress

The production of H_2O_2 by mitochondria gives an indication about the propensity of mitochondria to originate and/or exacerbate oxidative stress. In the absence of inhibitors of mitochondrial respiratory chain activity, the levels of H_2O_2 generated by rat heat mitochondria respiring glutamate/malate, expressed in nmol H_2O_2 mg^{-1} protein 15 min^{-1}, were low (2.61 ± 1.0). However, in the presence of 1 μM rotenone (an inhibitor of complex I) or 10 μM antimycin A (an inhibitor of complex III) the levels of H_2O_2 significantly increased to 8.07 ± 1.89 and 34.13 ± 10.71, respectively. As a control, we used catalase (643 U/ml) that dismutates H_2O_2 to H_2O plus O_2, to inhibit H_2O_2 production (with Antimycin A) from 34.13 ± 10.71 to 3.76 ± 0.77.

We analysed the effect of sildenafil citrate on the generation of H_2O_2 (Fig. 6) by mitochondria respiring glutamate/malate in the absence of inhibitors of the mitochondrial respiratory chain activity (Basal) and in the presence of rotenone (Rot) or antimycin A (AA). Sildenafil citrate significantly decreased the generation of H_2O_2 by mitochondria under all the experimental conditions tested. The generation of H_2O_2 by mitochondria respiring



Sil (μM)	Δψ (mV)			Phosphorylation Time (s)
	Glu/Mal Energisation	ADP Depolarisation	Repolarisation	
0	-211.7±2.3	23.0±2.9	-211.0±0.8	21.8±1.6
10	-212.2±1.8	23.9±3.4	-210.8±0.4	22.8±1.2
20	-213.0±2.5	24.7±2.7	-210.3±0.5	23.3±1.4
30	-211.9±2.2	23.5±3.1	-211.0±0.8	22.5±0.9
50	-211.5±2.1	22.7±3.2	-210.3±0.5	23.3±0.8

Fig. 3 Effect of sildenafil citrate on malate/glutamate-dependent transmembrane potential (ΔΨ) and phosphorylation time of rat heart mitochondria. Freshly isolated mitochondria were incubated at 0.5 mg/ml under standard conditions, as described in the "Materials and methods" section. Reactions were started by adding mitochondria. The traces are typical recordings representative of three experiments obtained from different mitochondrial preparations. Trace **a**, mitochondria in the absence of sildenafil (Sil); Trace **b**, mitochondria in the presence of 50 μM Sil. Where indicated by arrows, 0.5 mg mitochondria (Mit); and 200 μM ADP were added. The table underneath shows the mean ± SE of membrane potential, and phosphorylation time at the different indicated situations

glutamate/malate decreased 43% by 50 μM sildenafil citrate, while for mitochondria inhibited with rotenone or antimycin A the percentages were 41% and 30%, respectively.

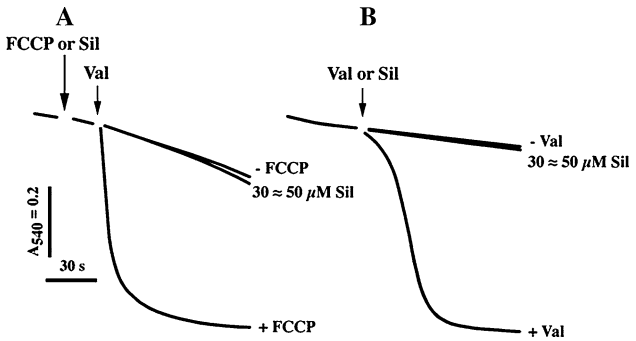


Fig. 4 Effect of sildenafil citrate on the permeabilisation to H^+ and K^+ by the inner membrane of rat heart mitochondria, evaluated by passive osmotic swelling of mitochondria suspended in K-acetate (**a**) and KNO_3 (**b**) media, respectively. Freshly isolated heart mitochondria were incubated at 0.5 mg/2.5 ml of appropriate medium, as described in the "Materials and methods" section. Where indicated by arrows, 1 μM FCCP; 30 and 50 μM sildenafil citrate (Sil); and 1 μg/ml valinomycin (Val) were added. The traces are typical recordings representative of three experiments obtained from different mitochondrial preparations

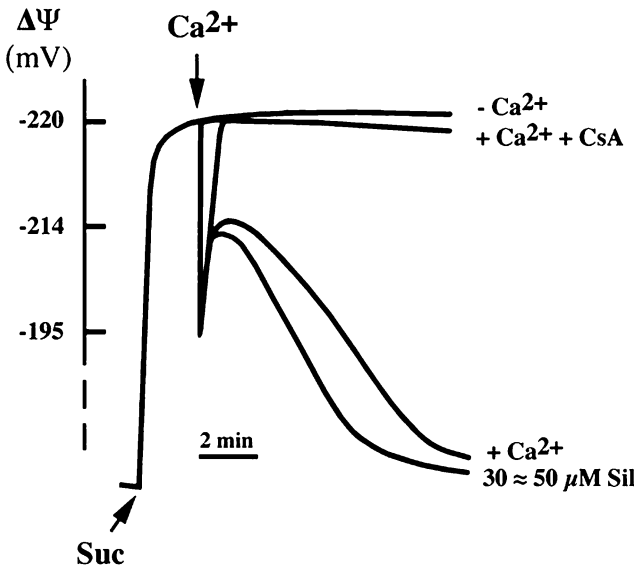


Fig. 5 Effect of sildenafil citrate (Sil) on Ca^{2+}-induced MPT evaluated by measuring mitochondrial transmembrane potential (ΔΨ) with a TTP^+ electrode. Freshly isolated heart mitochondria (0.5 mg) were incubated as described in the "Materials and methods" section. Mitochondria were energised with 10 mM succinate (Suc), and 300 nmol Ca^{2+} were added, where indicated, to induce MPT. One micromolar cyclosporin A (CsA) was used to confirm MPT induction. The traces are typical recordings representative of three experiments obtained from different mitochondrial preparations

Depression of H_2O_2 production by sildenafil citrate could be a result of its capacity to scavenge $O_2^{\bullet-}$, the primary ROS generated by the mitochondrial respiratory chain activity [16, 17]. In order to test this hypothesis we compared the capacities of sildenafil citrate and SOD to inhibit the reduction rate of NBT induced by the $O_2^{\bullet-}$ generated in a hypoxanthine/xantine oxidase system (Fig. 7). In the presence of the $O_2^{\bullet-}$ generating system, sildenafil citrate concentrations of up to 50 μM inhibited the reduction rate of NBT in a concentration dependent manner, indicating that the compound possesses the capacity to scavenge $O_2^{\bullet-}$. Considering the relationship between the SOD-like activity of sildenafil citrate versus its concentration (Fig. 7, inset), 50 μM sildenafil citrate has a SOD-like activity corresponding to about 0.85 units of SOD. This is a result that proves antioxidant capacity for sildenafil citrate, acting as an $O_2^{\bullet-}$ scavenger.

Discussion

In this study, we analysed the effects of sildenafil citrate on several heart mitochondrial parameters to correlate known actions of the compound with effects on heart mitochondria.

One of the main findings of your study was that sildenafil citrate concentrations of up to 50 μM did not affect heart mitochondrial bioenergetics, as shown by the lack of

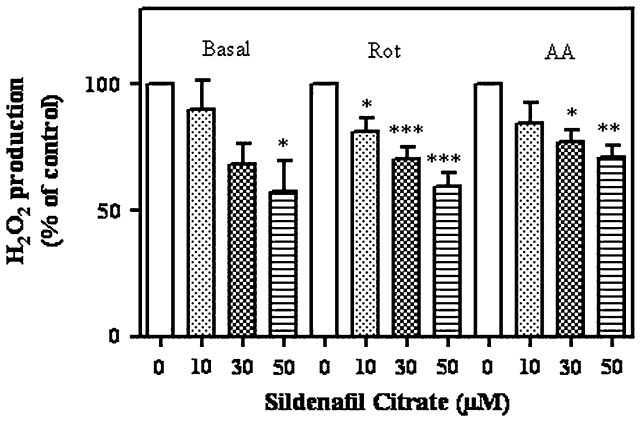


Fig. 6 Effect of sildenafil citrate on hydrogen peroxide (H_2O_2) production by rat heart mitochondria. Freshly isolated heart mitochondria were incubated at 0.2 mg/1.5 ml in standard conditions as described in the "Materials and methods" section. Reactions were started by adding 10 mM glutamate/5 mM malate and stopped 15 min later by adding cold glycine buffer. Basal, assays in the absence of mitochondrial respiration inhibitors; Rot, assays in the presence of 1 μM rotenone; AA, assays in the presence of 10 μM antimycin A. The results of H_2O_2 detection are given as a percentage (%) of the control value (mitochondria not treated with sildenafil citrate) of the respective experimental condition tested. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ statistically significant when compared to the control (100%). Control values, expressed in nmol H_2O_2 mg^{-1} protein 15 min^{-1}: Basal = 2.61 ± 1.0; Rot = 8.07 ± 1.89; AA = 34.13 ± 10.71. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ statistically significant when compared to the control value of the respective experimental condition tested. The results correspond to the mean ± SE of three experiments obtained from different mitochondrial preparations

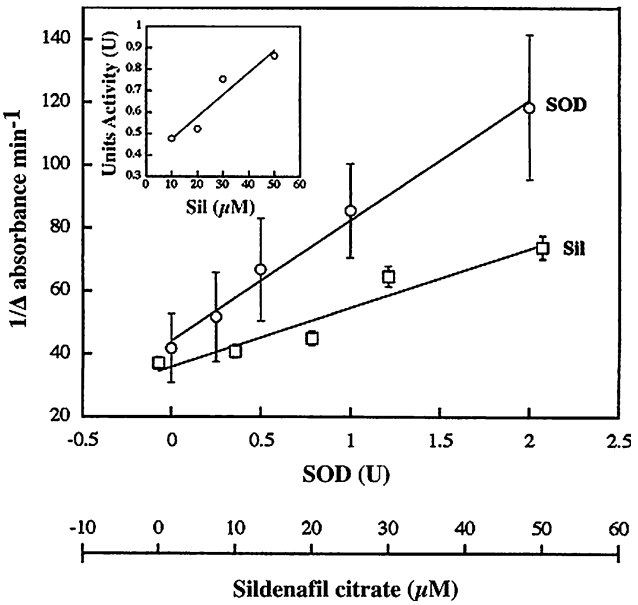


Fig. 7 Comparative effects of sildenafil citrate (Sil) and superoxide dismutase (SOD) on the reduction rate of nitroblue tetrazolium (NBT) by the superoxide radical ($O_2^{\bullet-}$) generated in a xanthine/xantine oxidase system. The assays were performed as described in the "Materials and methods" section. The rate of reduction of NBT, inhibited by SOD or Sil, is plotted as the reciprocal absorbancy change per minute versus units of SOD activity, or concentration of Sil, respectively. Inset: relationship between the SOD-like activity of sildenafil citrate, and sildenafil citrate concentration. The SOD-like activity of sildenafil citrate, for each concentration of sildenafil citrate, was calculated using the equation for the linear fit of the curve of SOD, $y = 44.171 + 38.22x$, with a correlation coefficient, $R = 0.99512$, meaning that 99% of the dependent variable variation ($1/\Delta$ absorbancy min^{-1}) is explained by the independent variable [SOD (U)]

effects on glutamate/malate-supported respiration in states 2, 3, 4, oligomycin inhibited state 3, FCCP-uncoupled respiration, RCR and ADP/O ratio (Fig. 2). Consequently, the $\Delta\Psi$ and the phosphorylation rate were not affected (Fig. 3). In agreement with these results, we also observed the inability of the compound to affect nonselective mitochondrial inner membrane permeabilisation to H^+ and K^+ (Fig. 4), indicating that sildenafil citrate concentrations of up to 50 μM do not impair rat heart mitochondrial function. The observation that 50 μM of the compound did not affect MPT (Fig. 5) indicates that the compound did not interfere with heart mitochondrial Ca^{2+} homeostasis.

Another main finding was that sildenafil citrate (10, 30, and 50 μM) protects heart mitochondria against oxidative stress, as revealed by its ability to depress H_2O_2 generation (Fig. 6). This can be a result of its SOD-like activity, decreasing $O_2^{\bullet-}$ production by mitochondria and, in doing so, avoiding H_2O_2 generation. Indeed, like SOD, sildenafil citrate inhibited the reduction rate of NBT induced by $O_2^{\bullet-}$ in a concentration dependent manner (Fig. 7). However, the SOD-like activity of sildenafil citrate cannot be attributed to dismutation of $O_2^{\bullet-}$ to H_2O_2, as it is by SOD, since we observed a decrease, instead of an increase, in the H_2O_2 generated by mitochondria. The hypothesis that the compound affects the activity of xantine oxidase enzyme cannot be clarified, because sildenafil citrate and ureate's spectra overlap at 292 nm [27].

Regardless of the precise mechanism by which sildenafil citrate depressed the H_2O_2 generated by heart mitochondria, the results presented in this study are well in line with previous findings showing that the compound exhibits an antioxidant effect, thus playing an important role in the protection against oxidative stress. It prevents cadmium- and lead-induced oxidative stress in the submandibular gland and saliva of rats [28, 29] and pulmonary embolism-induced oxidative stress in the plasma of dogs and rats, as well as in perfused lung preparations isolated from rats [30]. It also reduces diabetes-induced oxidative stress of plasma in streptozotocin-treated rats [31]. Furthermore, sildenafil citrate inhibits $O_2^{\bullet-}$ formation induced by the thromboxane A_2 analogue U46619, nicotine and tumor necrosis factor-α in cultured corpus cavernosal smooth muscle cells derived from rabbit penis [6, 32]. It also inhibits $O_2^{\bullet-}$ formation in isolated corpus cavernosum from hypercholesterolaemic rabbits [33] and pig pulmonary artery endothelial cells [34].

The concentrations of sildenafil citrate with antioxidant action in isolated heart mitochondria (Fig. 6) cannot be related to the protective concentrations used against ischemia/reperfusion injury in cardiomyocytes [18] and in isolated working heart model [35], because the conditions used and the parameters evaluated are different.

Like sildenafil citrate, other clinically approved PDE5 inhibitors (vardenafil and tadalafil), induce cardioprotective effects against ischemia/reperfusion injury [9, 11, 36–38]. It is believed that both sildenafil and vardenafil limit myocardial infarction through opening of the mitochondrial KATP channels [11, 36, 38], but the possibility of the same mechanism of action to be involved in the tadalafil effect is less clear [37]. In fact, the molecular structures of vardenafil and sildenafil are similar as compared with that of tadalafil [3, 7]. Nevertheless, it is difficult to foresee if these two compounds have similar or different effects to those described, in this study, for sildenafil citrate, because their influences on mitochondrial function and oxidative stress have not been studied.

In conclusion, at concentrations that did not affect heart mitochondrial bioenergetics and Ca^{2+}-induced permeability transition, sildenafil citrate protects heart mitochondria against H_2O_2 generation by acting as a SOD-mimetic. Heart ischemia induces increased generation of ROS, and subsequent reperfusion could result in toxic ROS overproduction that possibly contributes to irreversible damage of mitochondrial function and consequent impaired recovery of physiological function and cell death [13–15, 39, 40]. Hence, the antioxidant capacity of sildenafil citrate might be relevant to protect heart mitochondria against oxidative stress induced by ischemia/reperfusion injury and it may, putatively, contribute to its cardioprotective action. Moreover, mitoK_{ATP} channels act as ROS sensors capable of regulating ROS release in response to changes in mitochondrial redox-state [41]. In isolated heart, brain and liver mitochondria it was shown that conditions that increase mitoK_{ATP} activity decrease ROS release [42]. According to these statements, the possibility of sildenafil citrate to contribute to mitoK_{ATP} opening through changes in the mitochondrial redox status cannot be excluded.

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