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Inflammation Research

Role of NO/cGMP/K_{ATP} pathway in antinociceptive effect of sildenafil in zymosan writhing response in mice

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Abstract. *Objective:* Previous studies have found that sildenafil produces antinociception in experimental models. This work was undertaken to determine the participation of the NO/cGMP/K_{ATP} pathway in the antinociception induced by sildenafil.
Methods and results: The antinociceptive effect of sildenafil was determined in the zymosan-induced writhing response in mice. Sildenafil (1–30 mg/kg; i. p.), given 30 min before zymosan (1 mg/animal; i. p.), inhibited the writhing response (5.0 ± 1.3 versus 26.6 ± 2.7; $p < 0.001$) in a dose-dependent manner. L-NAME (30 mg/kg; s. c.) significantly ($p < 0.05$) reversed this effect (16.6 ± 3.1 versus 6.4 ± 1.6) and L-arginine (200 mg/kg; i. p.) prevented the L-NAME effect (6.8 ± 0.8 versus 16.6 ± 3.1; $p < 0.05$). ODQ (0,3–1 mg/kg; i. p.) and glybenclamide (0.3–1 mg/kg; p. o.) pre-treatment significantly ($p < 0.01$) inhibited the antinociceptive effect of sildenafil (18.0 ± 1.7 versus 2.1 ± 1.0 and 5.5 ± 0.7 versus 1.6+0.7, respectively). Diazoxide (10 mg/kg; s. c) significantly ($p < 0.001$) abolished the glybenclamide effect (1.6 ± 0.8 versus 14 ± 1.2).
Conclusions: The data indicate that the antinociceptive effect of sildenafil is dependent on the activation of the NO/cGMP/K_{ATP} pathway.

Introduction

Sildenafil is a selective and potent inhibitor of cGMP-specific phosphodiesterase (PDE5) which catalyzes the hydrolysis of cGMP and has a relaxant effect on the smooth muscle cells of the arterioles supplying the human corpus cavernosum [1, 2]. Previous studies have consistently found that sildenafil produces antinociception in the formalin test in rats [3, 4] and in the writhing response induced by acetic acid in mice [5, 6]. Mixcoatl-Zecuatl et al. [3] and Asomoza-Espinosa et al. [4] have suggested that sildenafil produces its antinociceptive effect through the accumulation of cyclic GMP as a consequence of phosphodiesterase 5 inhibition. Jain et al. suggested that sildenafil causes antinociception through the activation of the NO-cyclic GMP pathway [5, 6].

NO and cyclic GMP can activate several targets including different types of K^+ channels [7–9]. Soares et al. have recently shown in the rat paw pressure test that NO donor- and dibutyryl cyclic GMP-induced peripheral antinociception is reversed by tolbutamide and glybenclamide (ATP-sensitive K^+ channel blockers) [10, 11], thus establishing a link between the NO–cyclic GMP pathway, opening of K^+ channels, and antinociception. Recently, Sachs et al. [12] demonstrated that analgesic stimulators of the neuronal arginine/NO/cGMP/PKG/K_{ATP} pathway constitute a class of peripheral analgesics with a mechanism of action different from either glucocorticoids or cyclooxygenase inhibitors. The role of this pathway in sildenafil-induced antinociception has not been studied extensively and requires further clarification.

Based on the above considerations, this work was undertaken to determine the possible participation of the cyclic NO/cGMP/K_{ATP} pathway in antinociception induced by sildenafil. For this purpose, we tested the actions of L-NAME (non-selective NOS inhibitor), alone or with L-arginine (a NOS substrate), 1-1H-(1,2,4)-oxadiazolo(4,2-a)quinoxalin-1-one (ODQ, a guanylyl cyclase inhibitor), and glybenclamide (an ATP-sensitive K^+ channel blocker) alone or with diazoxide (an activator of K_{ATP} channels) in sildenafil-induced antinociception in the zymosan writhing response in mice.

Methods

Animals

Male Swiss mice (25–30 g) were housed in a temperature-controlled room, with access to water and food ad libitum and 12-h dark–light cycles until use. All experiments were conducted in accordance with National Institute of Health guidelines on the welfare of experimental animals and with the approval of the Ethics Committee of the School of Medicine of the Federal University of Ceará.

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Nociceptive test

Writhing test

Nociceptive activity was tested in mice using the writhing model [13]. The nociceptive stimulus, zymosan (1 mg mouse^{-1}; 0.2 ml), was injected into the peritoneal cavity of mice which were kept in a large glass cylinder, and the intensity of nociception was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection [14, 15]. The writhing response consists of the contraction of the abdominal muscle together with a stretching of the hind limbs.

Experimental Protocol

Determination of antinociceptive effect of sildenafil in zymosan-induced writhing in mice

Mice were treated with vehicle (saline; S) or sildenafil (1–10 mg kg^{-1}) and 20 min later, zymosan (1 mg mouse^{-1}; 0.2 ml) was injected. The number of writhes was counted as described above.

Effect of pretreatment with L-NAME on antinociceptive activity of sildenafil in zymosan-induced writhing in mice

To examine the effect of L-NAME on sildenafil activity, animals were pretreated with L-NAME (20 mg/kg; s.c.), 30 min before sildenafil (10 mg/kg; i.p.) injection. Zymosan (1 mg/animal) was administered 20 min after sildenafil injection. A control experiment was performed in the same way, but L-arginine (200 mg/kg; i.p.) was given 5 min before L-NAME injection. In other groups of mice, L-arginine (200 mg/kg; i.p.) or L-NAME (20 mg/kg; s.c.) alone were also injected followed by saline injections before zymosan administration. The number of writhes was counted as described above.

Effect of ODQ pre-treatment on antinociceptive activity of sildenafil in zymosan induced writhing in mice

The soluble guanylyl cyclase inhibitor ODQ (0.1–1 mg/kg) was injected 30 min priror to sildenafil (10 mg/kg; i.p.) administration. Zymosan (1 mg/animal) was injected 20 min after sildenafil and the number of writhes was counted as described above.

Effect of glybenclamide and/or diazoxide pre-treatment on the antinociceptive activity of sildenafil in zymosan induced writhing in mice

Glybenclamide (0.1–1 mg/kg; p.o.) or saline was injected 45 min before sildenafil (10 mg/kg; i.p.) administration. After 20 min, zymosan was injected. Other groups of animals received diazoxide (1 mg/kg; i.p.) or vehicle before glybenclamide (1 mg/kg; p.o.) administration or diazoxide (1 mg/kg; i.p.) plus vehicle, both followed by the stimulus injection. Twenty minutes after stimulus injection, the number of writhes was counted as described above.

Drugs

The following drugs were used: zymosan A, N^G-L-nitro-arginine methyl ester (L-NAME), L-arginine, 1H-[1,2,4] -oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), glybenclamide, diazoxide (Sigma-Aldrich, St Louis, MO). Sildenafil citrate was kindly provided by Pfizer (Sandwich, Kent, U.K.). Zymosan, L-NAME and L-arginine where diluted in saline. ODQ was diluted in saline plus 4 % dimethyl sulfoxide (DMSO), glybenclamide in 0.002N NaOH plus 4 % dextrose and diazoxide in 1N NaOH and saline.

Statistical analysis

Results are presented as means ±S.E.M. of measurements made on at least 5–6 animals in each group. Differences between responses were evaluated by analysis of variance (ANOVA) followed by Bonferroni's test. Statistical differences were considered to be significant at $P < 0.05$.

Results

Dose-response curve of the effect of sildenafil on the writhing response induced by zymosan in mice

The intraperitoneal injection of zymosan (1 mg/mouse) induced a writhing response that was determined for 20 min following injection of the stimulus. The pretreatment of the mice with sildenafil (1–10 mg kg/mg, 20 min before stimulus) inhibited in a dose-dependent manner the zymosan-induced writhing response. All the doses of sildenafil significantly inhibited the nociceptive response, although the dose of 10 mg/kg produced the maximal effect, inhibiting nociception by 81.2 % ($p < 0.001$) (Fig. 1).

Effect of L-NAME pre-treatment on antinociceptive activity of sildenafil in zymosan-induced writhing response in mice

Pre-treatment with L-NAME significantly ($p < 0.05$) reversed the antinociceptive activity of sildenafil by 57.2 % when compared to the saline pre-treated group. L-Ar-

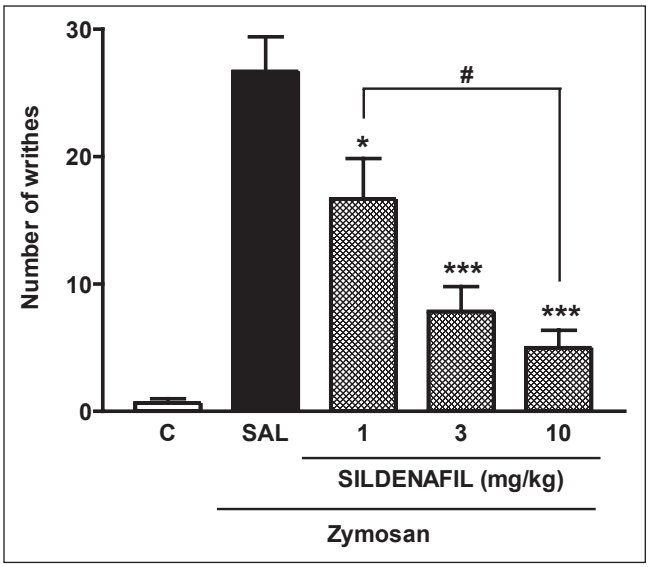

Fig. 1. Dose-response curve of the effect of sildenafil on zymosan-induced writhing activity in mice. The number of writhes was determined for the interval of 0 to 20 min, after i.p. injection of zymosan (1 mg/mouse). Sildenafil (1–10 mg/kg; i.p.) or vehicle (saline; SAL) was given 30 min before zymosan administration. C represents animals treated with i.p. saline without zymosan. Results are expressed as means ±S.E.M. for groups of six mice. Asterisks indicate statistically significant differences between groups and respective controls (*$P < 0.05$; ***$P < 0.001$) and # symbol indicates statistically significant differences between sildenafil (1 mg/kg) and sildenafil (10 mg/kg) groups.

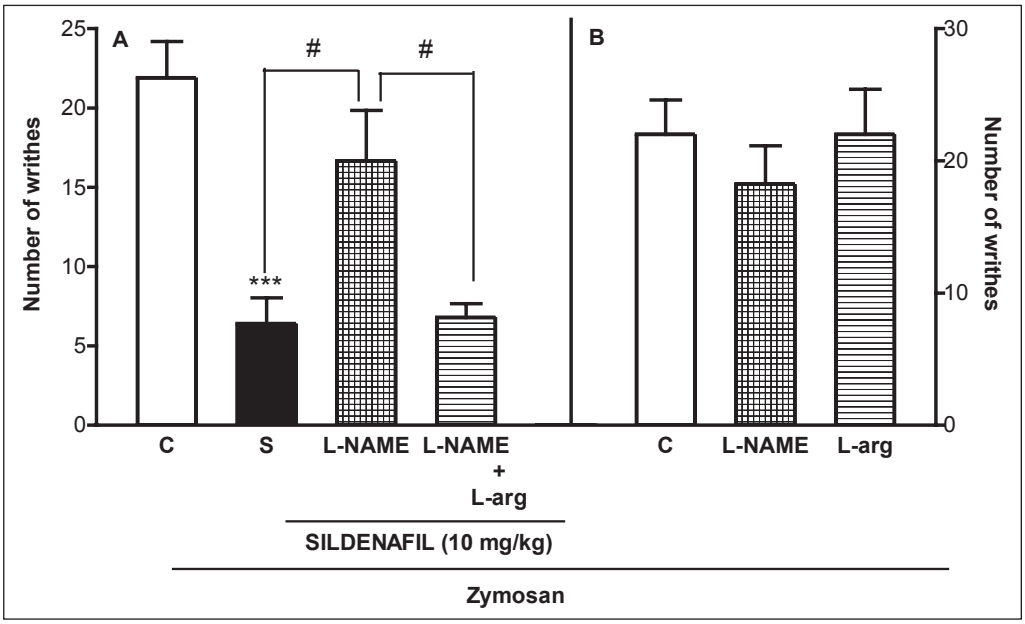

Fig. 2. Effect of L-NAME pretreatment on sildenafil antinociceptive activity in the zymosan-induced writhing response in mice. The number of writhes was determined for the interval of 0 to 20 min, after i. p. injection of zymosan (1 mg/mouse). Sildenafil (10 mg/kg; i. p.; S) or vehicle (saline; C) was given 30 min before zymosan administration. L-NAME (20 mg/kg; s. c.) was injected, 30 min before sildenafil (10 mg/kg; i. p.) injection. Zymosan (1 mg/animal) was administered 20 min after sildenafil injection, (panel A). A control experiment was performed in the same way, but giving L-arginine (200 mg/kg; i. p.) 5 min before L-NAME injection. In another group, L-arginine (200 mg/kg; i. p.) was also injected followed by 2 saline injections before zymosan administration, and another group received L-NAME (20 mg/kg; s. c.) preceded by 2 saline injections (Panel B). Results are expressed as means ±S.E.M. for groups of six mice. Asterisks indicate statistically significant differences between the vehicle-treated group (C) group and sildenafil group (S) (***P < 0.001), and # symbol indicates statistically significant differences between the L-NAME-treated group and others (#p < 0.05).

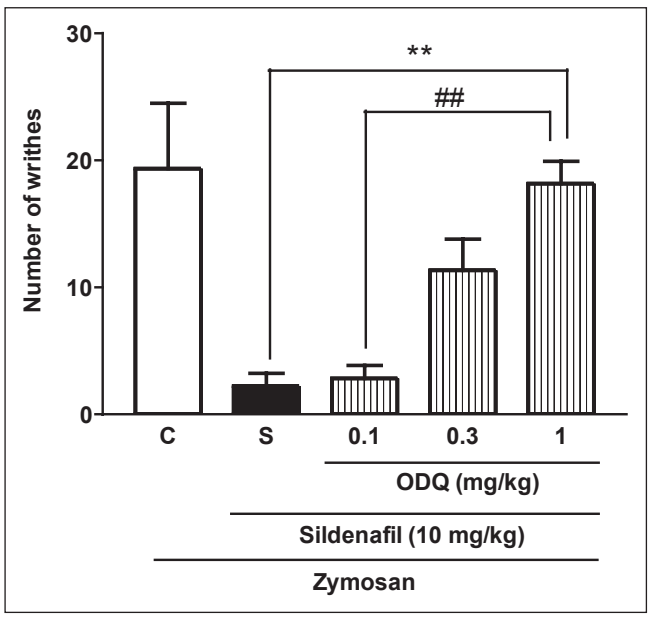

Fig. 3. Dose-response effect of ODQ on antinociceptive activity of sildenafil in zymosan-induced writhing response in mice. The number of writhes was determined for the interval of 0 to 20 min, after i. p. injection of zymosan (1 mg/mouse). Sildenafil (10 mg/kg; i. p.; S) or vehicle (saline; C) was given 30 min before zymosan administration. ODQ (0.1–1 mg/kg) was injected i. p., 30 min before sildenafil. Results are expressed as means ±S.E.M. for groups of six mice. Asterisks indicate statistically significant differences between the sildenafil pretreated group (S) and ODQ (1 mg/kg) group (**P < 0.01), and ## symbol indicates statistically significant differences between low dose ODQ (0.1 mg/kg) and high dose ODQ (1 mg/kg) (## p < 0.01).

ginine, given prior to L-NAME, inhibited this effect by 96 % and was significantly different ($p < 0.05$) when compared with the group that received saline plus L-NAME before sildenafil (Fig. 2, panel A). Neither of the groups pretreated only with L-NAME or only with L-arginine caused changes in the zymosan nociceptive effect (Fig. 2, panel B).

Effect of ODQ on antinociceptive activity of sildenafil on zymosan-induced writhing response in mice

Pre-treatment with ODQ (0.1–1 mg/kg) inhibited in a dose-dependent manner the antinociceptive effect of sildenafil on the zymosan-induced writhing response. Inhibition reached 95.5 % with a dose of 1 mg/kg and was significantly different ($p < 0.01$) from the group pretreated with saline plus sildenafil (Fig. 3).

Effect of glybenclamide and/or diazoxide on antinociceptive activity of sildenafil in zymosan-induced writhing in mice

Glybenclamide (0.1–1 mg/kg) reversed in a dose-dependent manner the antinociceptive effect of sildenafil. Reversal reached 74.07 %, with a dose of 1 mg/kg and was significantly different ($p < 0.01$) from the group pretreated with saline plus sildenafil (Fig. 4). Diazoxide, when given before glibencalmide (1 mg/kg), significantly ($p < 0.001$) abolished this effect by 87 %, and when given before sildenafil, without glybenclamide, significantly ($p < 0.001$) enhanced the antinociceptive effect of sildenafil by 97 % (Fig. 5).

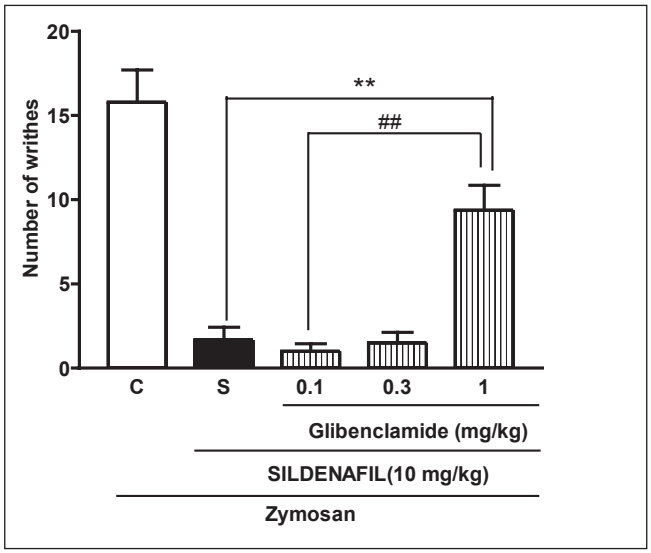

Fig. 4. Dose-response effect of glybenclamide pre-treatment on antinociceptive activity of sildenafil in zymosan-induced writhing response in mice. The number of writhes was determined for the interval of 0 to 20 min, after i. p. injection of zymosan (1 mg/mouse). Sildenafil (10 mg/kg; i. p, S.) or vehicle (saline; C) was given 30 min before zymosan administration. Glybenclamide (0.1–1 mg/kg) was administered, p. o., 45 min before silfenafil i. p. injection. Results are expressed as means ±S.E.M. for groups of six mice. Asterisks indicate statistically significant differences between the sildenafil pretreated group (S) and glybenclamide (1 mg/kg) group (**P < 0.01), and ## symbol indicates statistically significant differences between low dose glybenclamide (0.1 mg/kg) and high dose glybenclamide (1 mg/kg) (## p < 0.01).

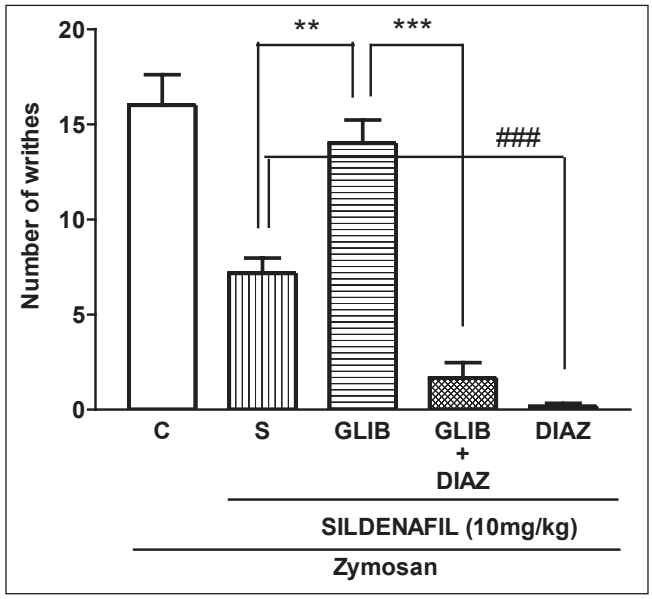

Fig. 5. Effect of diazoxide on glybenclamide inhibitory activity on antinociceptive outcome of sildenafil in zymosan-induced writhing. The number of writhes was determined for the interval of 0 to 20 min, after i. p. injection of zymosan (1 mg/mouse). Sildenafil (10 mg/kg; i. p., S.) or vehicle (saline; C) was given 30 min before zymosan administration. Glybenclamide (1 mg/kg) was administered, p. o., 45 min before silfenafil i. p. injection. After 20 min, zymosan was injected. Other groups of animals received diazoxide (1 mg/kg; i. p.) or vehicle before glybenclamide (1 mg/kg; p. o.) administration or diazoxide (1 mg/kg; i. p.) plus vehicle, both followed by the stimulus injection. Results are expressed as means S.E.M. for groups of six mice. Asterisks indicate statistically significant differences between the glybenclamide-treated group (GLIB) and adjacent groups (GLIB+DIAZ; S) (** P < 0.01, ***P < 0.001) and # symbol indicates statistically significant differences between the sildenafil-treated group (S) and diazoxide group (DIAZ; ###P < 0.001).

Discussion

The antinociceptive effect of sildenafil in experimental pain models has been described. Other authors have shown this effect in the writhing response induced by acetic acid [6] and in the formalin test in rats [3, 16]. The effect of sildenafil on PDE5 has been pointed as being responsible for its antinociceptive effect [5]. The NO/cGMP/PKG/KATP pathway has been reported as an additional mechanism of action of some peripheral analgesics such as diclophenac, dypirone, rofecoxib, ketorolac and central-acting drugs such as morphine [4, 6, 16]. Sachs et al. [12] have described this sequence of events that ends with the opening of potassium channels as a mechanism antagonistic to the hyperalgesic state that occurs in inflamed tissues. They proposed that the activation of this pathway should be used to promote analgesia, not only in acute pain, but also in chronic pain, as was demonstrated using the persistent hypernociceptive model [12]. In chronic pain, it seems that sensitive neurons acquire a pain memory that involves ion channels. K_{ATP} openers such as diazoxide and drugs which activate the NO/cGMP/PKG/ K_{ATP} pathway can block the hypernociception induced after a quiescent phase. The hypernociception induced after the quiescent phase (memory) is not blocked by COX inhibitors such as indomethacin. Although peripheral analgesics which activate the NO/cGMP/PKG/ K_{ATP} pathway can block ongoing hypernociception by restoring the normal threshold of the nociceptors, which is obtained by the opening of potassium channels. Thus, drugs that activate the NO/cGMP/ K_{ATP} pathway could represent a new approach for pain treatment. These findings taken together have motivated us to investigate if the antinociceptive effect of sildenafil is dependent on the NO/cGMP/ K_{ATP} pathway.

Concerning the importance of potassium channel opening to prevent nociception and also to antagonize ongoing nociception, as demonstrated for some peripheral-acting drugs and for sildenafil, our work provides novel data that support these hypotheses. The data shown in the present study suggest that sildenafil has an anti-nociceptive activity that is NO dependent, leading to the opening of ATP-sensitive K^+ channels, which may be the final event leading to the nociception inhibition. We propose that the mechanism involving increased cGMP levels can be explained by the PDE5 blockade and also by guanylyl-cyclase activation induced by NO release. Our hypothesis is based on the reversal of the antinociceptive activity of sildenafil by ODQ, a soluble guanylyl cyclase blocker. The activation of soluble guanylyl cyclase must be the result of the NO release induced by zymosan intraperitoneal injection. Consistent with our hypothesis, there is evidence that zymosan intraperitoneal injection induces NO production by peritoneal cells [18] and that the presence of NO is necessary for the antinociceptive effect of sildenafil, since L-NAME blocked this effect with no changes upon zymosan nociceptive activity. The release

of NO leading to guanylyl-cyclase activation is responsible for the cGMP generation which is enhanced by sildenafil blockade of PDE5. The activation of guanylyl cyclase is important to sildenafil effect since ODQ, the soluble guanylyl cyclase blocker, is effective in inhibit sildenafil antinociceptive activity. These findings involving NO release have also been demonstrated by other authors in other models [16]. This thinking is in agreement with the finding that sildenafil enhances the antinociceptive activity of NO-releasing drugs such as nitroprussiate and morphine. The authors who have shown a synergistic effect of morphine and sildenafil suggest that this synergistic effect must be due only to PDE5 block which shows synergism with the morphine mechanism of activation of the NO/cGMP pathway [3]. Other work shows that the synergistic effect of sildenafil/nitroprusside is blocked by methylene blue pretreatment [5] indicating a possible effect of sildenafil on cGMP production in the presence of NO. Mixcoatl-Zecuatl et al. [3] have reported that ODQ reverses the antinociceptive synergistic effect of sildenafil-morphine combination in the formalin test, but not morphine effect when administered alone, confirming that ODQ action is upon sildenafil effect. This is another evidence that sildenafil can enhance the cGMP production by other drugs that act by promoting NO release such as morphine

Other authors have suggested that the antinociceptive activity of sildenafil is due only to its potent effect on PDE5 [6]. They have demonstrated that the antinociceptive activity of sildenafil is not reversed by pre-treatment with ODQ, in the formalin test [3]. This phenomenon may be explained based on the model used and by the nociceptive stimuli, since other authors using the acetic acid writhing model have shown that methylene blue, an unspecific guanylyl-cyclase inhibitor, reverses the antinociceptive activity of sildenafil [17]. Another explanation of these findings can be that sildenafil increases the NO activity which was released by the stimulus, since zymosan can induce NO production by peritoneal resident cells [18].

We suggest that the opening of K_{ATP} channels is preceded by cGMP generation mediated by guanylyl cyclase which is activated by NO release, and that sildenafil may also increase cGMP accumulation by inhibiting PDE5 activity as well. We also suggest that, for the sildenafil antinociceptive effect, it is necessary that the NO production has been achieved.

Data from literature reports that the pathway that leads to the opening of K_{ATP} channels after cGMP generation may include the activation of PKG [10–12]. Recent report suggest that the antinociceptive effect of sildenafil on the formaline test may involve the activation of PKG following the increase of cGMP generation [16] and that the antiallodynic effect of spinal gabapentin is due to PKG activation via cGMP generation [19]. Our data do not provide an explanation about the involvment of PKG on sildenafil antinociceptive effect on the zymosan writhing model, although we suggest that probably the opening of K_{ATP} channel by sildenafil pretreatment is secundary of PKG activation. However, other authors have demonstrate that spinally delivered PKG inhibitors reduce formalin-induced nociceptive behavior in rats [20, 21], as well as that spinal PKG-I is involved in the facilitation of synaptic transmission of nociceptive stimuli in the spinal cord in an ongoing activation, whereas acute heat-induced nociception does not require PKG-I activity [22].

Taking these findings and our data into account, we suggest that the antinociceptive effect of sildenafil depends on the activation of NO production, which in turn activates the soluble guanylyl cyclase leading to cGMP accumulation which finally may open ATP-sensitive K^+ channels, possibly as a result of PKG phosphorylation. Inhibition of PDE5 activity by Sildenafil should further increase cGMP accumulation. Such a mechanism could contribute to its antinociceptive activity. We concede that this is just speculation on the mechanism of action for sildenafil in zymosan nociceptive activity, and that more data is needed to confirm this hypothesis.

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