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Scavenging of nitric oxide by an antagonist of adenosine receptors

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Abstract

Chronic treatment of rats with 1,3-dipropyl-8-sulfophenylxanthine (DPSPX), an antagonist of adenosine receptors, causes hypertension, cardiovascular hypertrophy and hyperplasia and impaired endothelium-dependent vasodilatation. An accelerated degradation of nitric oxide (NO) by scavenging molecules could account for endothelial dysfunction and trophic changes in this hypertension. Our aim was to determine whether DPSPX is a scavenger of NO and if this putative effect is shared by caffeine (1,3,7-trimethylxanthine) and DPCPX (1,3-dipropyl-8-ciclopentylxanthine), which are also adenosine receptor antagonists but do not induce hypertension in rats. This effect was evaluated by electrochemical and spectrofluorometric assays. Urinary NOx (nitrate + nitrite) excretion was also evaluated in controls and DPSPX-treated rats as a marker for NO bioavailability. DPSPX behaved as a scavenger of NO in a concentration-dependent manner in the electrochemical and spectrofluorometric assays. Caffeine and DPCPX had no scavenging effect. DPSPX-treated rats had decreased excretion of urinary nitrites. We can conclude that: DPSPX has NO scavenging properties that may be involved in the alterations described for DPSPX-hypertensive rats; this NO-scavenging effect is not shared by caffeine and DPCPX, which are also xanthine derivatives and adenosine antagonists.

Introduction

Prolonged treatment of rats with 1,3-dipropyl-8-sulfophenylxanthine (DPSPX), a water-soluble (Daly et al 1985), non-selective antagonist of A1/A2 adenosine receptors, causes a hypertension that lasts for at least seven weeks after the end of the administration of the drug (Albino-Teixeira et al 1991). This hypertensive state is accompanied by hypertrophic and hyperplastic alterations in the cardiovascular system (Albino-Teixeira et al 1991; Matias et al 1991; Morato et al 2003). DPSPX-hypertensive rats also present impaired endothelial function, demonstrated by a significant reduction in the vascular relaxation to carbachol (an endothelium-dependent relaxing substance) (Paiva et al 1997).

Nitric oxide (NO) is the main agent responsible for the relaxation of vascular smooth muscle cells (Furchgott & Zawadski 1980; Moncada et al 1988). The term endothelial dysfunction usually implies impairment of the endothelium-dependent vasorelaxation caused by the loss of NO vascular activity (Wennmalm 1994). Two main causes can contribute to the decline of NO bioactivity: a reduced production of NO by endothelial NO synthase (eNOS) and an accelerated consumption of NO. This degradation may be carried out by reactive oxygen species or by molecules with scavenging properties (Cai & Harrison 2000; Brodsky et al 2001). NO is a free radical and the presence of an odd electron in its structure confers a high reactivity. DPSPX may also have a high reactivity towards electrophilic compounds because it is negatively charged at physiological pH (Tofovic et al 1991), and contains a sulfonate group susceptible to interaction with NO.

When using drugs as tools to characterize physiological and pharmacological processes we should not exclude the putative direct effects of these drugs in such processes, besides their actions as an agonists or antagonists. Keeping this in mind, we aimed to evaluate whether DPSPX exhibits NO scavenging properties that could contribute to the endothelial dysfunction (Paiva et al 1997) and vascular hypertrophy and hyperplasia observed in DPSPX-treated rats (Albino-Teixeira et al 1991). We also aimed to determine whether this putative effect is shared by other xanthines, such as the non-selective A_1/A_2 adenosine receptors antagonist, caffeine (1,3,7-trimethylxanthine) and the selective A_1 adenosine receptors antagonist, DPCPX (1,3-dipropyl-8-ciclopentylxanthine), which are used as tools to clarify the role of adenosine in cardiovascular processes (Rudolphi et al 1989; Kilpatrick et al 2001) but do not induce hypertension in rats.

Materials and Methods

Drugs

All drugs were purchased from Sigma (St Louis, MO).

Electrochemical assay

NO concentration was measured using an NO electrode connected to an NO meter (ISO-NO Mark II; WPI, Sarasota, FL) and the data collected into a PC computer using the WPI interface. NO readily diffused through a gas-permeable polymeric membrane that covered the electrode. The oxidation of NO at the surface of the electrode generated an electrical current whose intensity was directly proportional to the concentration of NO. The selectivity of the NO-sensitive electrode was tested in connection with calibration, as previously described (He & Liu 2001). NO solutions were prepared by bubbling the purified gas through high-quality deionized water (obtained from a Milli-Q system; Millipore Company, Bedford, MA) degassed with argon. Before its utilization, the NO gas (>99.5% purity; Air Liquide, Paris, France) was purified in the absence of oxygen by consecutive passages through 10 M KOH in an apparatus using stainless-steel or Teflon tubing and glass containers. To evaluate the putative scavenging effect of DPSPX, caffeine or DPCPX on NO, a saturated solution of 2 μM NO was added to the reaction vial in the absence and in the presence of increasing concentrations (300 μM, 600 μM and 1 mM) of DPSPX or caffeine solutions in PBS, or DPCPX solutions in ethanol. The electrical currents generated by NO oxidation were recorded and the kinetic traces compared. Ethanol was also assayed to evaluate any possible interference of the solvent in DPCPX assay.

Spectrofluorometric assay

NO was generated by sodium nitroprusside. Sodium nitroprusside, in aqueous solutions, at physiological pH, generates NO only when exposed to light (Bates et al 1991; Ullrich et al 1997). NO production was measured by using the sensitive dye 4,5-diaminofluorescein (DAF-2). On reaction with NO, DAF-2 is converted to the highly fluorescent form triazolofluorescein, DAF-2T, the presence of which can be measured as a specific increase in fluorescence signal (Kojima et al 1998; Nagata et al 1999). Scavengers of NO compete with DAF-2, leading to a reduced production of fluorescence. DAF-2, sodium nitroprusside and caffeine were dissolved in phosphate-buffered solution (PBS) (KH_2PO_4 50 mM, pH 7.4). DPSPX was dissolved in dimethyl sulfoxide (DMSO). Immediately before the experiments, sodium nitroprusside and DAF-2 solutions were prepared and kept on ice, in the dark. DAF-2 solution (1300 μL, final concentration 3.14 μM) was then mixed in reaction vials with sodium nitroprusside (100 μL, final concentration 20 μM, 10 μM or 5 μM) and with PBS (100 μL) or DMSO (controls) or DPSPX (100 μL, final concentration 625 μM–10 mM) or caffeine (100 μL, final concentration 156 μM–5 mM). Under the conditions of this assay, it was not possible to test DPCPX in this range of concentrations, due to its insolubility in water solutions and reduced solubility in ethanol and DMSO. A reaction vial containing DAF-2 (1300 μL) and PBS (100 μL + 100 μL) without sodium nitroprusside was used to evaluate DAF-2 autofluorescence. The reaction vials were incubated for 10 min, in a water bath at 37°C, under light emission from a tungsten lamp. After this period, the vials were kept for 5 min in the dark, at 25°C. Then, the fluorescence of the solutions was measured at room temperature using a spectrofluorometer (LS-50B, Perkin-Elmer) with excitation wavelength at 495 nm and emission wavelength at 521 nm.

Measurement of urinary NOx (nitrate + nitrite) concentration

Housing and experimental treatment of the animals were conducted under the European Community guidelines for the use of experimental animals (European convention for the protection of vertebrate animals used for experimental and other scientific purposes, 1986, and Protocol of amendment to the European convention for the protection of vertebrate animals used for experimental and other scientific purposes, 1998).

Two groups of male Wistar rats, 250–300 g, were used: a group treated with DPSPX (90 $\mu g\,kg\,h^{-1}$) for 7 days and a control group. Alzet osmotic minipumps (model 2ML1; Alza, Palo Alto, CA), intraperitoneally implanted (day 0) under pentobarbital sodium anaesthesia (60 $mg\,kg^{-1}$, i.p.), were used for continuous infusion of DPSPX (90 $\mu g\,kg\,h^{-1}$, for 7 days). Alzet pumps were completely filled with DPSPX solution using a 3-mL syringe. The excess solution was wiped off and the flow moderator was fully inserted into the body of the pump. Pre-filled pumps were placed in 0.9% saline for 4–6 h before implantation to ensure constant flow of the fluid (Albino-Teixeira et al 1989). After the surgery, rats were housed in individual metabolic cages (Tecniplast, Buguggiate-VA, Italy) for 8 days. These metabolic cages featured a collection funnel and a separating cone design that effectively separated urine and faeces into tubes outside the cage. The rats were kept under regular photoperiod conditions (12-h light–dark cycle) at 23°C and 60% relative humidity. A 24-h urine collection was made on days 2, 4, 6 and 8. Urine samples were stored at −80°C until assayed. At the end of the study, rats were anaesthetized with pentobarbital sodium as before, and the osmotic minipumps were removed. The function of the pump was checked by visual inspection of the pump after longitudinal sectioning (collapsed lumen, measurement of remaining volume) (Albino-Teixeira et al 1989). The concentration of total NOx was evaluated by Griess reaction with potassium nitrite as a standard. Urine samples were diluted 5 fold with distilled water. One hundred microlitres of each sample was incubated for 30 min with 100 μL of nitrate reductase (43 mU mL^{-1}), 100 μL of FAD (35 μM), 100 μL of NADPH (0.28 mM) and 200 μL of potassium phosphate buffer (KH_2PO_4 0.1 M and K_2HPO_4 0.1 M, pH 7.2) at 37°C (Hosogai et al 2001). After addition of the Griess reagent (a 1:1 mixture of sulfanilamide 1% and 0.1% naphtylethylenediamine) for 10 min at room temperature, nitrites were measured by spectrophotometry at 540 nm.

Data analysis

In the electrochemical assay, the area under curve (AUC) was calculated to compare NO availability in the presence and in the absence of DPSPX, caffeine and DPCPX. These calculations were made by integration of the function y = f(x), where y is the electrical signal (pA) generated by NO and x represents time (s), using the Microcal Origin 6.1 software. AUC was expressed as pA s.

NO measurements in the spectrofluorometric assay were expressed as % of the fluorescence of control vials (DAF-2 + sodium nitroprusside + PBS). The IC50 value for DPSPX was calculated using Graph Pad Prism 3.0 analysis for sigmoidal concentration–effect curves.

Statistical analysis of the data (expressed as mean ± s.e.m.) obtained in the electrochemical and spectrofluorometric assay was carried out by analysis of variance, followed by Newman–Keuls test. Analysis of data obtained in the NOx measurement was performed by unpaired Student's *t*-test. $P < 0.05$ was considered significant.

Results

Electrochemical assay

DPSPX induced a significant decrease in the magnitude of the NO-generated signal, as shown by the values of the AUC obtained for each experimental situation (Figure 1). No significant differences were observed in the presence of caffeine or DPCPX (Figure 2).

Spectrofluorometric assay

The fluorescence produced by NO-induced DAF-2 oxidation was significantly decreased by DPSPX in a concentration dependent manner (Figure 3). This scavenging effect over NO was also dependent on sodium nitroprusside concentration. In the presence of sodium nitroprusside 20 μM the IC50 of DPSPX was 4.82 ± 0.15 mM. The IC50 of DPSPX significantly decreased for lower concentrations of sodium nitroprusside (2.67 ± 0.29 mM for sodium nitroprusside 10 μM ($P < 0.05$ vs 20 μM) and 0.78 ± 0.07 mM for sodium nitroprusside 5 μM ($P < 0.05$ vs 10 μM and 20 μM)); results are means ± s.e.m, n = 4–5; no effect was observed in the presence of caffeine (data not shown).

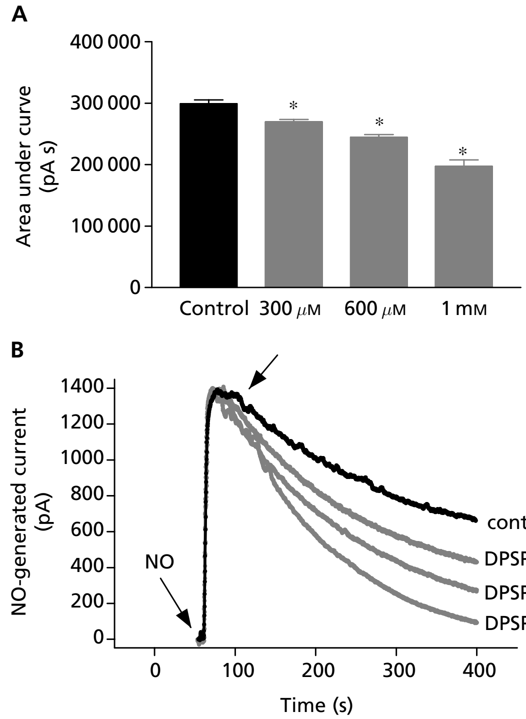


Figure 1 Effect of DPSPX on the amount of NO measured in the electrochemical assay. A. Area under curve corresponding to the NO-generated curve in the presence of DPSPX. *$P < 0.05$ vs control (analysis of variance, followed by Newman–Keuls test), n = 5–8. B. Typical experimental trace following DPSPX addition. The y-axis refers to the electrical signal (pA) generated by NO. The first arrow indicates the addition of NO to the reaction vial and the second one indicates addition of DPSPX.

Measurement of urinary NOx concentration

DPSPX-treated rats had decreased excretion of urinary nitrites from day 4 to day 8, when compared with the control group (Figure 4).

Discussion

This study clearly demonstrates that DPSPX is a scavenger of NO. In the electrochemical assay a significant reduction of the NO-generated current was observed immediately after DPSPX addition to the reaction vial. The scavenging effect was also confirmed by the spectrofluorometric assay where DPSPX decreased the fluorescence produced by NO-induced oxidation of DAF-2. This effect was dependent on DPSPX concentration for both assays.

The interaction between DPSPX and NO is not surprising, as they are reactive species. The chemical nature of NO, and especially its reactivity towards thiol groups and

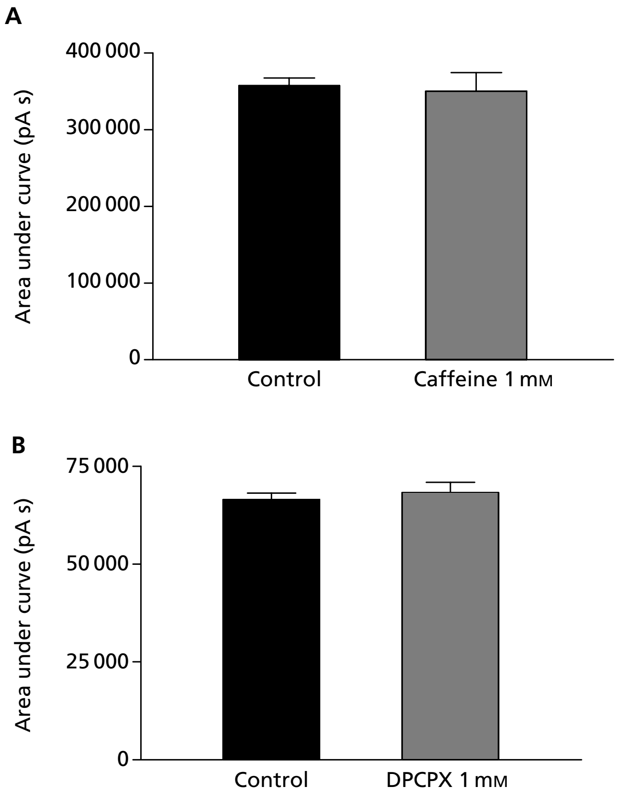


Figure 2 Area under curve corresponding to NO-generated curve in the presence of caffeine (A) or DPCPX (B).

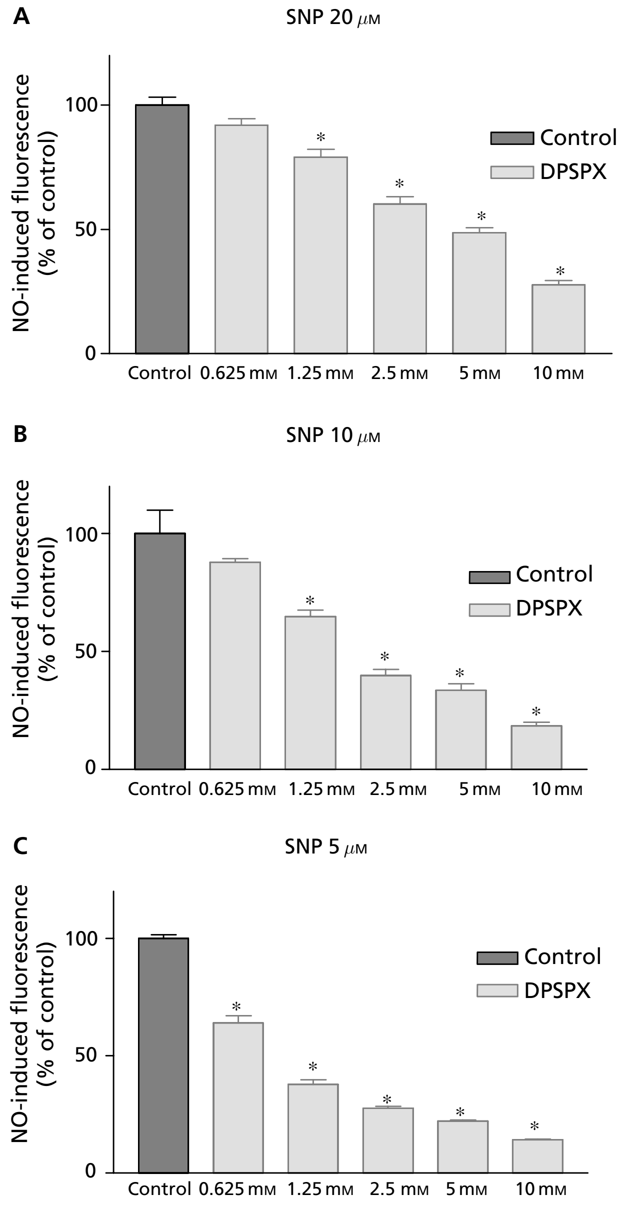


Figure 3 Effect of DPSPX on the fluorescence produced by the reaction of NO with DAF-2. NO was generated from sodium nitroprusside (SNP) 20 μM (A), 10 μM (B) or 5 μM (C). NO-induced fluorescence is expressed as % of control. Values are means $\pm$ s.e.m., n = 4–5. *$P < 0.05$ vs control.

other sulfur-containing compounds, could be an explanation for its reaction with 1,3-dipropyl-8-sulfophenylxanthine. In fact, it has been reported that NO reacts with a benzenesulfonate compound (3,5-dibromo-4-nitrosobenzenesulfonate, DBNBS), which is used to detect nitrites in biological samples (Nazhat et al 1999). Both DPSPX and DBNBS have a benzenesulfonate group, which may be responsible for the reactivity toward NO. This interpretation is further supported by the evidence that other xanthines (caffeine, DPCPX) that do not contain a sulfonate group in their structure (Figure 5), had no effect on NO-induced signal under the conditions of our assays. The lack of scavenging effect of NO by caffeine and DPCPX is of great importance since these drugs have been extensively used as pharmacological tools to provide evidence of the protective role played by adenosine in experimental models of ischaemia/hypoxia and anoxia reperfusion (Rudolphi et al 1989; Sutherland et al 1991; Lasley et al 1992; Neely et al 1996; Kilpatrick et al 2001). If these adenosine antagonists had a scavenging effect on NO it would be difficult to differentiate the protection conferred by adenosine from the protection exerted by NO, since NO also has the potential to exhibit beneficial effects in ischaemic tissue (Williams et al 1995; Imagawa et al 1999).

DPSPX scavenging of NO may contribute to the alterations described for the DPSPX model of hypertension. Since DPSPX is negatively charged at physiological pH, it is unable to penetrate cell membranes and to be metabolized by the liver (Tofovic et al 1991). Thus, DPSPX only distributes in the extracellular space and may reach high levels in the plasma, when infused chronically. It has already been described that an intravenous infusion of DPSPX ($10\,mg + 150\,\mu g\,min^{-1}$) generates serum levels of DPSPX of 120–140 $\mu g\,mL^{-1}$ during the first 80 min of the infusion (Tofovic et al 1991), which is equivalent to serum concentrations of 300–350 μM.

In this work we have observed that there is a positive correlation between the sodium nitroprusside (NO donor) concentration and the IC50 for the scavenging effect of DPSPX. So, in conditions where NO bioavailability is

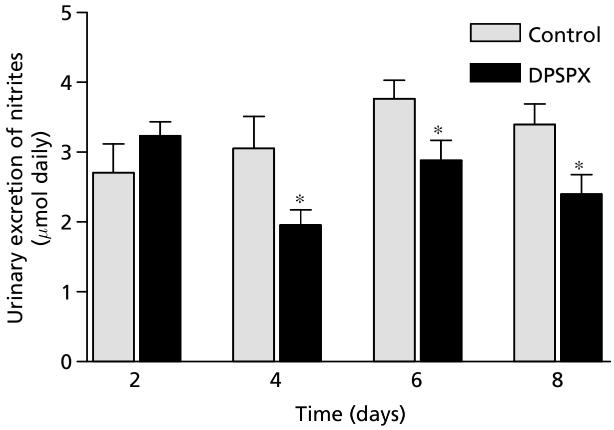
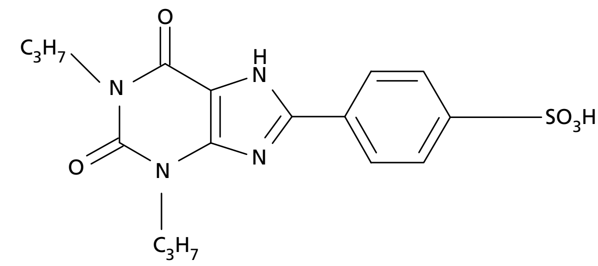


Figure 4 Twenty-four-hour urinary nitrite and nitrate (NOx) excretion in controls and DPSPX-treated rats on days 2, 4, 6 and 8. Results are shown as mean ± s.e.m.; n = 7–8; $^*P < 0.05$ vs control.

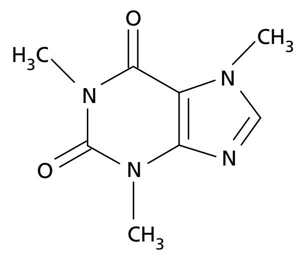
decreased (e.g. hypertension, oxidative stress) DPSPX may have a higher scavenging effect over NO and may worsen the consequences of the impairment of NO bioactivity. Urinary NOx concentrations are used as markers of total body NO generation and bioavailability. In this study DPSPX-treated rats had decreased levels of urinary nitrites from day 4 to day 8, which is in accordance with our finding of an NO-quenching effect by DPSPX. On day 2 we observed no significant differences between the nitrite concentrations of controls and DPSPX-treated rats, but this result may be due to a surgery-induced inflammation that can increase NO levels (Kalff et al 2000).

The consequences of NO bioactivity impairment may include deficient endothelium-dependent vasodilatation, which is a hallmark of experimental and human hypertension, and the loss of the NO regulatory role of vascular smooth muscle cell proliferation (Ignarro et al 2001), thus contributing to vascular hyperplasia. DPSPX-treated rats have impaired endothelium-dependent vasodilatation (Paiva et al 1997), marked cardiovascular hypertrophy and hyperplasia (Albino-Teixeira et al 1991; Matias et al 1991; Morato et al 2003), renin–angiotensin system activation (Morato et al 2002; Sousa et al 2002) triggered by the blockade of adenosine receptors, and changes in oxidant and antioxidant enzymes (Sousa et al 2004a, b). Although the relative contribution of the toxicological and pharmacological effects of DPSPX, namely nitric-oxide scavenging, inhibition of adenosine modulation of the cardiovascular system, activation of the renin–angiotensin system and oxidative stress activation, still need to be elucidated with respect to the cardiovascular changes observed in DPSPX-treated rats, it is possible that all these mechanisms act in a synergistic manner to maintain the hypertensive state.

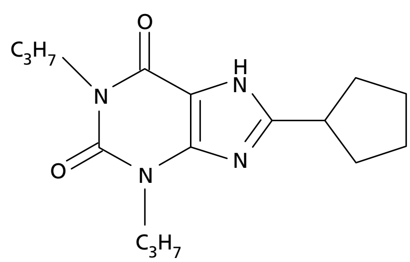
We can conclude that DPSPX is an NO scavenger. The reduction of NO availability, mediated by a direct scavenging effect of DPSPX, may contribute to the endothelial dysfunction (Paiva et al 1997) and vascular hypertrophy and hyperplasia (Albino-Teixeira et al 1991; Morato et al 2003) observed in DPSPX-hypertensive rats. Although



1,3-Dipropyl-8-sulfophenylxanthine
(DPSPX)



1,3,7-Trimethylxanthine
(Caffeine)



1,3-Dipropyl-8-ciclopentylxanthine
(DPCPX)

Figure 5 Chemical structures of DPSPX, caffeine and DPCPX.

this NO-scavenging effect is not shared by caffeine or DPCPX, which are also xanthine derivatives and adenosine antagonists, a similar scavenging effect should be expected and considered, when interpreting results, for other sulfoxanthine derivatives (Fahim et al 2001), also used as tools to clarify the role of adenosine in the cardiovascular system.

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