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-cyclopentylxanthine), 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 nitrates. 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 watersoluble (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 A1/A2 adenosine receptors antagonist,
caffeine (1,3,7-trimethylxanthine) and the selective A1
adenosine receptors antagonist, DPCPX (1,3-dipropyl-
8-cyclopentylxanthine), 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 hyper-
tension 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 pro-
portional to the concentration of NO. The selectivity of the
NO-sensitive electrode was tested in connection with cali-
bration, as previously described (He & Liu 2001). NO solu-
tions were prepared by bubbling the purified gas through
high-quality deionized water (obtained from a Milli-Q sys-
tem; 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 M NO was added to the reaction vial in the absence
and in the presence of increasing concentrations (300 M,
600 M and 1 nm) 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 fluo-
rescent form triazolofluorescein, DAF-2T, the presence
of which can be measured as a specific increase in fluores-
cence 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) (KH2PO4 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 M, final concentration
3.4 M) was then mixed in reaction vials with sodium
nitroprusside (100 M, final concentration 20 M, 10 M
or 5 M) and with PBS (100 M) or DMSO (controls) or
DPSPX (100 M, final concentration 625 M–10 mm) or caffeine
(100 M, final concentration 156 M–5 mm). Under the condi-
tions 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 M) and PBS (100 M + 100 M) without sodium
nitroprusside was used to evaluate DAF-2 autofluores-
cence. 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 spectrophotometer
(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 protec-
tion 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 M kg kg–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 M kg kg–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 con-
stant 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 meta-
abolic 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 anaesthe-
tized 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 μM mL−1), 100 μL of FAD (35 μM), 100 μL of NADPH (0.28 mM) and 200 μL of potassium phosphate buffer (KH₂PO₄ 0.1 M and K₂HPO₄ 0.1 M, pH 7.2) at 37°C (Dosogai et al 2001). After addition of the Griess reagent (a 1:1 mixture of sulfanilamide 1% and 0.1% naphthylethylenediamine) 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 μM. The IC50 of DPSPX significantly decreased for lower concentrations of sodium nitroprusside (2.67 ± 0.29 μM for sodium nitroprusside 10 μM (\( P < 0.05 \)) vs 20 μM) and 0.78 ± 0.07 μM 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).

**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
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 μg min⁻¹) generates serum levels of DPSPX of 120–140 μg 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

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

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**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 ± s.e.m., n = 4–5. *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 nitrates 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 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.

References


