Xanthine Oxidase Inhibition by 1,3-dipropyl-8-sulfophenylxanthine (DPSPX), an Antagonist of Adenosine Receptors

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Xanthine oxidase (XO), an enzyme involved in purine metabolism, is a source of either oxidants (superoxide radical) or antioxidants (uric acid). Interference with XO activity can lead to oxidative stress, thus contributing to the pathogenesis of cardiovascular diseases. The adenosine receptors antagonist, 1,3-dipropyl-8-sulfophenylxanthine (DPSPX), induces hypertension and cardiovascular injury in rats. Since DPSPX is a xanthine, we aimed at evaluating DPSPX’s influence on XO activity to ascertain its contribution to DPSPX-induced hypertension. The activity of isolated XO in the presence of DPSPX was evaluated spectrophotometrically. Serum and urinary uric acid levels of DPSPX-treated rats were measured using a commercial kit. DPSPX inhibited XO activity in a concentration-dependent manner and reduced rat serum and urinary uric acid levels. It can be concluded that: DPSPX is an inhibitor of XO; decreased generation of uric acid may lead to oxidative stress, thus contributing to endothelial dysfunction and vascular morphological changes in DPSPX-treated rats.

Keywords: Xanthine oxidase; DPSPX; Hypertension; Uric acid; Oxidative stress; 1,3-dipropyl-8-sulfophenylxanthine

INTRODUCTION

Chronic treatment of rats with 1,3-dipropyl-8-sulfophenylxanthine (DPSPX), a water soluble, non selective antagonist of adenosine receptors, causes a long lasting hypertensive state for at least seven weeks even after the administration of the drug is ceased. This hypertensive state is accompanied by hypertrophic and hyperplastic alterations in the cardiovascular system. Since adenosine exerts a physiological brake upon renin release, the antagonism of adenosine receptors can enhance the activity of the renin-angiotensin system. This pressor system is activated in this model, as shown previously by the higher plasma renin activity and higher angiotensin II plasma levels, thus contributing to the development and maintenance of the hypertension induced by DPSPX. This is further supported by the fact that angiotensin-converting enzyme inhibition and angiotensin II receptor antagonism prevented the rise in blood pressure and the alterations in cardiovascular morphology.

However, when using a drug as a pharmacological tool in order to characterise physiological and pathophysiological processes, the putative direct effects of the drug in such processes should also be considered, besides its receptor mediated effects. Xanthine oxidase is an enzyme involved in purine metabolism and has been considered to play an important role in the pathogenesis of oxidant-induced microvascular changes and tissue injury because the enzyme reaction that transfers electrons from hypoxanthine to uric acid is coupled with a reduction of molecular oxygen into superoxide radicals. There is also evidence that superoxide radical derived from xanthine oxidase might alter nitric oxide bioavailability in spontaneously hypertensive rats (SHR), leading to increased arteriolar tone.

Furthermore, the reaction of superoxide with NO yields peroxynitrite (ONOO−), a highly reactive...
intermediate with cytotoxic properties that can react with a variety of biomolecules including proteins, lipids and DNA. Peroxynitrite is involved in the pathogenesis of atherosclerosis, ischemia-reperfusion injury, and hypertension.

Besides the production of pro-oxidant molecules, xanthine oxidase also generates uric acid, which is an important antioxidant agent that scavenges peroxynitrite and hydroxyl radical. Microvascular endothelium and myocardium have been shown as major sites of uric acid production. Furthermore, uric acid prevents oxidative inactivation of endothelial enzymes and increases the stability of the antioxidant ascorbate.

Interference with xanthine oxidase activity can result in an imbalance in antioxidant/oxidant production, either by increasing the production of reactive oxygen species (ROS) or by decreasing antioxidant defences.

Since DPSPX is structurally a xanthine, an interaction with xanthine oxidase could be expected to occur. This study is aimed at evaluating the interaction between DPSPX and xanthine oxidase in order to ascertain its putative contribution to the hypertensive state and cardiovascular injury observed in DPSPX-treated rats.

MATERIALS AND METHODS

Reagents

DPSPX was obtained from Sigma-RBI. Xanthine and xanthine oxidase grade I from buttermilk (EC 1.1.3.22), were purchased from Sigma. All reagents were of analytical grade.

Evaluation of Superoxide Radical Production During the Putative DPSPX Metabolism by XO

The production of superoxide radical by XO was determined spectrophotometrically in a 96 well plate reader (Ceres 900) by monitoring the effect of DPSPX on the reduction of cytochrome c by O$_2^{-}$ at 550 nm. The reaction mixtures in the sample wells consisted of DPSPX (44 μM), XO (0.0483 unit/ml), and cytochrome c (50 μM) in a final volume of 300 μl.

DPSPX and cytochrome c were dissolved in phosphate buffer 50 mM with EDTA 0.1 mM, pH 7.8, and xanthine oxidase was dissolved in EDTA 0.1 mM, pH 7.8. The reaction was conducted at room temperature for 2 min and initiated by the addition of XO.

Effect of DPSPX on XO Activity (Uric Acid Measurement)

The effect of DPSPX on XO activity was evaluated by measuring the formation of uric acid from xanthine in a double-beam spectrophotometer (Shimadzu 2600) at room temperature. The assay mixture contained: 44 μM xanthine, xanthine oxidase (0.0483 unit/ml), DPSPX (10, 20, 30, 40, 50 or 75 μM), 50 mM phosphate buffer with 0.1 mM EDTA, pH 7.8, in a final volume of 600 μl. The absorbance was measured at 295 nm during 2 min. Additionally, this procedure was repeated with several concentrations of xanthine (11, 22 and 44 μM) and DPSPX (25 and 50 μM), to get the inhibitory type (Lineweaver-Burk plots) for DPSPX.

Calculation of Percent Inhibition of XO Activity

Percent inhibition = (ΔA$_{\text{test}}$/ΔA$_{\text{control}}$) × 100,

where ΔA$_{\text{control}}$ is the absorbance change/min in the control test and ΔA$_{\text{test}}$ is the absorbance change/min in the sample tests.

Calculation of IC$_{50}$

The IC$_{50}$ value was calculated from regression lines corresponding to 4 experiments performed in triplicate, where the x axis was the tested compound and the y axis was percent inhibition of enzyme activity.

In Vivo Studies

Male Wistar rats (250–300 g) were used. The animals were kept under regular photoperiod conditions (12 h dark, 12 h light) at 23°C temperature and 60% relative humidity. Standard laboratory rat chow and water were available ad libitum. Three groups of rats were used: the control group received an infusion of saline i.p., the second group was treated with DPSPX (90 μg kg$^{-1}$) for 3 days and sacrificed on day 3 (3 d) and the third group received an infusion of DPSPX (90 μg kg$^{-1}$) for 7 days and was sacrificed on day 14 (14 d). Alzet osmotic minipumps (model 2ML1; Alza, Palo Alto, CA, U.S.A.), intraperitoneally implanted (day 0) under pentobarbitone sodium anaesthesia (60 mg kg$^{-1}$, i.p.), were used for continuous infusion of DPSPX (90 μg kg$^{-1}$) or saline (vehicle). Systolic and diastolic blood pressure were measured by the tail-cuff method (LE 5000, Letica, Barcelona, Spain) in conscious animals. Five determinations were made each time during the training period, on day 0 and at the end of the study, and the means used for further calculation.

Uric Acid Determination in Serum and Urine Samples

Serum Uric Acid

Rats were anaesthetized with pentobarbitone sodium (60 mg kg$^{-1}$, i.p.). Blood was withdrawn
from the left ventricle into ice cold tubes, incubated at 37°C for 30 min and immediately centrifuged (3000 rpm, 10 min, 4°C). Serum samples were stored at −80°C until assayed for uric acid. Uric acid present in the samples of control, DPSPX 3d and DPSPX 14d groups was measured spectrophotometrically (520 nm) by an enzymatic assay using a commercially available kit (ABX Diagnostics Uric Acid PAP 80).

**Urinary Uric Acid**

Male Wistar rats (250–300 g) were divided into control and DPSPX (90 μg kg⁻¹, i.p., for 7 days)-treated rats, and then housed in individual metabolic cages (Tecniplast, Buguggiate-VA, Italy) for 14 days, which allowed the separate collection of urine and faeces. The animals were kept under regular photoperiod conditions (12 h dark, 12 h light) at 23°C temperature and 60% relative humidity. A 24 h- urine collection was made on day 3, for controls and DPSPX-treated rats. Urine samples were stored at −80°C until uric acid assay. The uric acid present was measured spectrophotometrically (520 nm) by an enzymatic assay using a commercially available kit (ABX Diagnostics Uric Acid PAP 80).

**Data Analysis**

All values are expressed as mean ± s.e.m. Statistical analysis of the data was carried out by analysis of variance (ANOVA) and followed by Newman-Keuls test. P values of less than 0.05 were considered significant.

**RESULTS**

**Evaluation of Superoxide Radical Production:**

DPSPX did not behave as a xanthine oxidase substrate since it was not observed to produce any O₂⁻ formation.

**Inhibition of XO by DPSPX:**

DPSPX inhibited the activity of XO in a concentration-dependent manner (Figure 1), as measured by a decrease in uric acid generation. The IC₅₀ of DPSPX was 51.77 ± 1.12 μM.

**Lineweaver-Burk Plots**

To evaluate the inhibitory mechanism of DPSPX, xanthine oxidase activity was tested at different substrate concentrations (Figure 2). In the presence of DPSPX, both Vₘₐₓ (enzyme activity) and Kₘ (apparent substrate affinity) were lowered (Table I), suggesting a mixed noncompetitive inhibition, which means that DPSPX binds both to the enzyme and to the xanthine–xanthine oxidase complex, but with greater affinity for the latter.

**Arterial Blood Pressure**

Infusion of DPSPX increased systolic and diastolic blood pressure in the 3 d and 14 d groups (Figure 3).

**Uric Acid Measurements**

Serum uric acid (mg/dl) was significantly decreased in the 3 d group when compared with the control. The 14 d group (that received DPSPX for 7 d and was sacrificed on day 14) was not different from the control (Figure 4).

Urinary excretion of uric acid (mg/24 h) was also significantly decreased in rats treated with DPSPX for 3 days (Figure 5) when compared with control animals.

![FIGURE 1](image-url)  
**FIGURE 1** The inhibitory effect of DPSPX on xanthine oxidase activity. Values show mean ± s.e.m. from 4 experiments performed in triplicate.

![FIGURE 2](image-url)  
**FIGURE 2** Lineweaver-Burk plots in the absence (control) and in the presence of DPSPX 25 μM with xanthine as the substrate. Values show mean ± s.e.m. from three experiments performed in triplicate. V = ΔA/min; S = xanthine (μM).

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<th>[DPSPX] (μM)</th>
<th>inhibition of XO activity (%)</th>
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<tr>
<td>0</td>
<td>0</td>
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<td>25</td>
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<td>50</td>
<td>50</td>
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<td>75</td>
<td>75</td>
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<td>100</td>
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**TABLE I** Vₘₐₓ (enzyme activity) and Kₘ (apparent substrate affinity) for xanthine oxidase in the absence (control) and in the presence of DPSPX 25 or 50 μM

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<tr>
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<th>Kₘ (μM)</th>
<th>Vₘₐₓ (ΔA/min)</th>
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<tr>
<td>Control (xanthine)</td>
<td>142.86</td>
<td>0.3702</td>
</tr>
<tr>
<td>Xanthine + DPSPX 25 μM</td>
<td>76.92</td>
<td>0.2025</td>
</tr>
<tr>
<td>Xanthine + DPSPX 50 μM</td>
<td>47.62</td>
<td>0.1084</td>
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DISCUSSION

This study demonstrates that DPSPX is a potent xanthine oxidase inhibitor, acting in the micromolar range. This was confirmed both "in vitro" by measuring the activity of isolated xanthine oxidase in the presence of different concentrations of DPSPX, and "in vivo" by measuring the serum uric acid (a product of purines metabolism by xanthine oxidase) in rats treated with DPSPX. The decrease in serum uric acid in the group treated for 3 days is in accordance with a direct effect of DPSPX on xanthine oxidase because, after the end of the DPSPX infusion, the uric acid level returns to control values. Urinary excretion of uric acid is also decreased in the group treated for 3 days, which is in agreement with the lower values of serum uric acid being due to diminished production and not to an uricosuric effect of DPSPX.

Xanthine oxidase is an ubiquitous enzyme that catalyses the hydroxylation of many purine substrates and converts hypoxanthine to xanthine and then xanthine to uric acid, in the presence of molecular oxygen yielding superoxide radical.7,10 Xanthine oxidase-derived superoxide radical has been linked to endothelial dysfunction 20 (due to the loss of nitric oxide bioactivity), to postischemic tissue injury and edema.21,22 Superoxide readily reacts with nitric oxide, yielding peroxynitrite, a potent oxidant involved in lipid peroxidation and cell death.12 If DPSPX behaved as a substrate of xanthine oxidase, an increase of superoxide radical and peroxynitrite production could be expected and could be involved in DPSPX-induced vascular injury, namely loss of endothelium vasodilation23 and vascular hypertrophy.1 As DPSPX is an inhibitor of xanthine oxidase activity, an increase of superoxide radical generation by this metabolic pathway should not be expected. However, in DPSPX-hypertensive rats there are changes in antioxidant markers, namely an increase in mesenteric artery glutathione peroxidase activity in the acute and chronic phase of this hypertension, suggesting alterations of the redox state.24 Antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase) and other antioxidant molecules such as uric acid, ascorbate, glutathione, function as a cooperative system that counteracts the oxidant effects of ROS and reactive nitrogen species (RNS), that can be responsible for cell injury. An imbalance in oxidant/antioxidant production can lead to oxidative stress, a condition that has been associated with the development of many diseases (hypertension, atherosclerosis, ischemia reperfusion injury).25–27

Although inhibition of xanthine oxidase by DPSPX does not account for an increased production of an oxidant molecule, it does contribute to the reduction of antioxidant capacity, by inhibiting uric acid generation. Serum and urinary uric acid are significantly decreased during DPSPX infusion. Uric acid has been proven to be a potent antioxidant capable of scavenging ROS and RNS like peroxynitrite and hydroxyl radicals.16,17 The microvascular endothelium is a major site of uric acid production,28 and the release of uric acid constitutes an important antioxidant defense in the coronary system.16
It has been described that chronic inhibition of uric acid formation by allopurinol decreases coronary dilatation by acetylcholine in guinea pig perfused heart, and that exogenous application of uric acid prevents the impairment of vascular responses to acetylcholine upon generation of superoxide radical. Decrease of uric acid content caused by generation of superoxide radicals by this enzyme, inactivation and in the activation of growth-related signaling pathways. In conclusion, our findings indicate that DPSPX is a potent inhibitor of xanthine oxidase. This inhibition may be involved in the vascular injury observed in its hypertensive effect, since it leads to decreased levels of an important antioxidant scavenger.

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References
