Research Paper

Tempol improves xanthine oxidoreductase-mediated vascular responses to nitrite in experimental renovascular hypertension

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Upregulation of xanthine oxidoreductase (XOR) increases vascular reactive oxygen species (ROS) levels and contributes to nitroso-redox imbalance. However, XOR can generate nitric oxide (NO) from nitrite, and increased superoxide could inactivate NO formed from nitrite. This study tested the hypothesis that XOR contributes to the cardiovascular effects of nitrite in renovascular hypertension, and that treatment with the antioxidant tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) improves XOR-mediated effects of nitrite. Blood pressure was assessed weekly in two-kidney one-clip (2K1C) and control rats. After six weeks of hypertension, the relaxing responses to nitrite were assessed in aortic rings in the presence of the XOR inhibitor oxypurinol (or vehicle), either in the absence or in the presence of tempol. Moreover, in vivo hypotensive responses to nitrite were also examined in the presence of oxypurinol (or vehicle) and tempol (or vehicle). Aortic XOR activity and expression were evaluated by fluorescence and Western blot, respectively. Vascular ROS production was assessed by the dihydroethidium assay. 2K1C hypertensive rats showed increased aortic XOR activity and vascular ROS production compared with control rats. Oxypurinol shifted the nitrite concentration–response curve to the right in aortic rings from 2K1C rats (but not in controls). Oxypurinol also attenuated the hypotensive responses to nitrite in 2K1C rats. Tempol treatment enhanced oxypurinol-induced shift of the nitrite concentration–response curve to the right. However, antioxidant treatment did not affect XOR-mediated hypotensive effects of nitrite. Our results show that XOR is important to the cardiovascular responses to nitrite in 2K1C hypertension, and XOR inhibitors commonly used by patients may cancel this effect. This finding suggests that nitrite treatment may not be effective in patients being treated with XOR inhibitors. Moreover, while tempol may improve the vascular responses to nitrite, antihypertensive responses are not affected.

1. Introduction

Xanthine oxidoreductase (XOR) is a molybdoflavine enzyme that catalyzes the oxidation of hypoxanthine to xanthine, and xanthine to uric acid in the metabolic pathway of purine degradation [1, 2]. It exists in two interconvertible forms: xanthine dehydrogenase (XDH), which is the prevalent form under physiological conditions in vivo [3], and xanthine oxidase (XO), which is derived from XDH by posttranslational modifications [4]. Increased production of superoxide by XOR increases tissue levels of this free radical and other reactive oxygen species (ROS), thus contributing to nitroso-redox imbalance in several disease conditions including hypertension [5–7]. Indeed, upregulated vascular XOR activity has been shown in animal models of hypertension including spontaneous hypertensive rats (SHR) [8] and deoxycorticosterone-salt (DOCA-salt) hypertension [9].

In addition to its prooxidant properties, XOR converts nitrite anion into nitric oxide (NO) under particular circumstances, thus contributing to the beneficial effects of this vasoactive NO metabolite [10–12]. In this regard, nitrate and nitrite are now described as the major sources of NO independent of the classical enzymatic NO formation from l-arginine [13–16]. Although they were initially
2. Materials and methods

2.1. Animals and hypertension model

The present study was performed in accordance with National Institutes of Health (NIH; USA) guidelines. All experimental protocols with animals were approved by our Institutional Animal Care and Use Committee of the Ribeirao Preto Medical School, University of Sao Paulo. Male Wistar rats (180–250 g) were obtained from the colony at the University of Sao Paulo (Ribeirao Preto Campus, Brazil) and were maintained on a 12-h light/dark cycle at room temperature (22 ± 2 °C) with free access to standard rat chow and water.

Hypertension (two-kidney, one clip; 2K1C) was induced as previously described [30,31]. Briefly, the rats were anesthetized with ketamine (100 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.) and the left renal artery was clipped with a silver clip (0.2 mm). As normotensive controls, sham operated rats underwent the same surgical procedure without placement of the renal artery clip. Hypertension was considered when blood pressure (BP) exceeds the value obtained in sham-operated rats. BP was measured weekly in anesthetized (100 mg/kg ketamine; 10 mg/kg xylazine; i.p.) rats using tail-cuff plethysmography [30,31]. To minimize the effects of stress induced by this method on blood pressure measurement, the animals were trained for a week before surgery. The rats were considered hypertensive when BP > 160 mmHg two weeks after the surgery [30,31].

2.2. Assessment of xanthine oxidoreductase activity

Plasma and aortic XOR activity was measured with a horse-radish peroxidase–linked commercial kit (Cayman Chemical Co., Ann Arbor, MI, USA) following the manufacturer’s instructions. In brief, plasma and aorta homogenates were added to working solution containing ADP (10-μmol/l) or vehicle. The re-
catheters were then tunneled subcutaneously and exteriorized through the back of the neck. Flunixin meglumine (2.5 mg/kg, sc, Banamine; Schering Plough, Brazil) was administered for post-operative analgesia. After 12 h of fasting, the arterial cannula was connected to a pressure transducer and the MAP was recorded in freely moving rats using a data acquisition system (MP150CE; Biopac Systems Inc. CA, USA) connected to a computer (Acknowledgment 3.2, for Windows). We then examined the hypotensive responses to intravenously infused sodium nitrite (1, 3, 10, 30, and 100 μmol/kg) in sham and 2K1C rats that received vehicle (NaOH 0.15 mol/l; 25 μl/min) or oxypurinol (25 mg/kg; 25 μl/min) intravenously 15 min before the nitrite infusions started [20]. Each cumulative doses of sodium nitrite was infused every 15 min, and the maximum changes in MAP were evaluated.

2.5. Assessment of vascular reactive oxygen species (ROS) production

To assess vascular oxidative stress, aortic ROS production was measured by using dihydroethidium (DHE), which is a ROS-sensitive fluorescent dye. Aortic cryosections (5 μm thick) were incubated with DHE (10 μmol/l) for 30 min at room temperature. The sections were examined by fluorescence microscopy (Leica Imaging Systems Ltd., Cambridge, England) at ×400 using λ = 525 nm excitation and λ = 605 nm emission, which is not specific to detect only superoxide. Red fluorescence from 20 fields around the vessel was evaluated using image software imageJ [http://rsbweb.nih.gov/ij/] as previously described [33]. In some experiments, the aortas were pretreated for 1 h with tempol (10⁻⁴ mol/l, a superoxide dismutase analogue), or diphenyl iodonium (DPI 10 μmol/l), a flavoprotein inhibitor, or Tiron (1 mmol/l, a superoxide scavenger).

2.6. Functional studies to examine whether reduction in oxidative stress enhances XOR-mediated vascular responses to nitrite

Given that oxidative stress associated with hypertension decreases NO bioavailability NO [6, 28, 29], we examined whether reduction of oxidative stress with tempol increases the vascular responses to nitrite that are mediated by XOR. Using the same methodology as detailed above, aortic rings from 2K1C rats were incubated with the tempol (10⁻⁴ mol/l) and the oxypurinol (3 × 10⁻⁴ mol/l) or vehicle (control experiments). After preconstriction with phenylephrine (10⁻⁷ mol/l), relaxing response curves to cumulative concentrations (from 3 × 10⁻⁸ mol/l to 10⁻² mol/l) of sodium nitrite were constructed. Finally, to assess whether oxidative stress affects the vasorelaxing effects of sodium nitrite mediated by XOR, we compared the changes in pD2 produced by oxypurinol (pD2 with vehicle minus pD2 with oxypurinol) in aortic rings from 2K1C rats in the presence and the absence of tempol.

In addition, we used the same experimental conditions detailed above to examine whether reduction of oxidative stress with tempol improves the contribution of XOR to the hypotensive effects of sodium nitrite. Therefore 2K1C hypertensive rats received tempol (18 mg/kg) by gavage [34] and, 15 min later, they received infusions of vehicle (NaOH 0.15 mol/l; 25 μl/min) or oxypurinol (25 mg/kg; 25 μl/min) for 10 min. After 15 min, the rats received cumulative doses of sodium nitrite (1, 3, 10, 30, and 100 μmol/kg) intravenously. Nitrite doses were injected every 15 min, and the maximum changes in MAP were evaluated. We then compared the area under the curve (AUC) of the changes in MAP induced by sodium nitrite in the presence or in the absence of tempol and oxypurinol (or vehicle).

2.7. Drugs and solutions

All drugs and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA) and all solutions were prepared immediately before use.

2.8. Statistical analysis

The results are expressed as means ± SEM. The comparisons between groups were assessed by unpaired t test or by Mann Whitney test, and by two-way analysis of variance, using Bonferroni correction where appropriated (GraphPad Prism Software, San Diego, CA). A probability value < 0.05 was considered significant.

3. Results

3.1. Renovascular hypertension increases vascular XOR activity

As expected, SBP increased in 2K1C rats compared with sham rats (189 ± 30 versus 119 ± 22 mmHg at the sixth week of hypertension; P < 0.05). Because XOR is an important pro-oxidant enzyme, we examined whether 2K1C hypertension increases vascular and circulating XOR activity. We found increased XOR activity in plasma (Fig. 1A; 227 ± 3 versus 206 ± 5 μU/ml; P < 0.05) and in aortas (Fig. 1B; 97 ± 24 versus 32 ± 9 μU/μg protein; P < 0.05) from 2K1C rats compared with sham rats. Control experiments using the XOR inhibitor oxypurinol showed that this inhibitor blunted > 85% of vascular XOR activity measured in aortic rings from 2K1C rats (Fig. 1C; P < 0.05). The analysis of XOR expression in the aortas showed only a trend for increased aortic XOR expression in 2K1C compared to sham rats (Fig. 1D and E; not significant).

3.2. Xanthine oxidoreductase contributes to the vasodilatory and antihypertensive effects of sodium nitrite in renovascular hypertension

Because we observed increased plasma and vascular XOR activity in 2K1C rats, we examined whether increased XOR activity would enhance the vasodilatory responses to nitrite, which is converted into NO by XOR. Indeed, incubation of aortic rings from 2K1C rats with the XOR inhibitor oxypurinol shifted the concentration-effect curve in response to sodium nitrite to the right (Fig. 2A and B; pD2 vehicle = 4.2 ± 0.2 versus pD2 oxypurinol = 3.7 ± 0.1; P < 0.05) without significantly changing the maximum effect (Fig. 2A and C; Emax vehicle = 98 ± 2% versus Emax oxypurinol = 98 ± 12%; P > 0.05). However, in contrast with aortic rings from 2K1C rats, XOR inhibition with oxypurinol produced no change in pD2 (Fig. 2A and B; vehicle = 4.3 ± 0.1 versus oxypurinol = 4.4 ± 0.1; P > 0.05) and in Emax (Fig. 2A and C; vehicle = 95 ± 1%, versus oxypurinol = 96 ± 1%; P > 0.05) values in aortic rings from sham-operated animals, thus suggesting that XOR contributes to the vasodilatory effects of sodium nitrite in 2K1C hypertension, but not in normotensive sham animals.

To further validate the role of XOR in the responses to nitrite therapy, we investigated in vivo implications of increased XOR activity in 2K1C hypertension. Blood pressure responses to intravenous sodium nitrite were assessed in sham and 2K1C rats after XOR inhibition with oxypurinol or vehicle treatment. Under control conditions (vehicle treatment), sodium nitrite produced dose-dependent hypotension in unanesthetized freely moving rats, both in normotensive and hypertensive rats (Fig. 3A and B; P < 0.05). However, while pretreatment with oxypurinol had no effects on the hypotensive responses to nitrite in sham-operated animals (Fig. 3A; P > 0.05), it attenuated those responses in 2K1C hypertensive rats, particularly when the highest nitrite dose (100 μmol/kg) was infused (Fig. 3B; −26 ± 4 mmHg for vehicle-treated versus −8 ± 2 mmHg for oxypurinol-treated; P < 0.05). These results suggest an important role for XOR in the hypotensive responses to nitrite in 2K1C hypertensive rats, and agree with the results found with isolated vessels.
3.3. Treatment with tempol improves XOR-dependent vasodilatory but not the hypotensive responses to nitrite in 2K1C hypertension

The vascular production of ROS was examined in aortas by using the sensitive fluorescent dye dihydroethidine (DHE). In agreement with previous studies [5,31,32], we found a 4-fold increase in ROS levels in aortas from 2K1C as compared with sham rats (Fig. 4A and B; $P < 0.05$). Quantification of DHE fluorescence in control experiments using aortas from hypertensive rats showed that the superoxide dismutase analogue tempol, or the flavoprotein inhibitor DPI, or the superoxide scavenger tiron, blunted the DHE signal by $80\%$ (Fig. 4B and C; $P < 0.05$).

Because oxidative stress reduces NO activity [6,28,29], XOR-derived superoxide may readily inactivate nitrite-derived NO [28] and impair the contribution of XOR to the vasodilator effects of sodium nitrite. We used aortas from 2K1C hypertensive rats to assess how the antioxidant tempol modifies XOR-mediated vasodilatory effects of sodium nitrite shown in Fig. 2. Interestingly, experiments carried out in the presence of tempol showed that pretreatment with oxypurinol shifted the concentration-effect curve in response to sodium nitrite to the right (Fig. 5A and B; $pD_2$ tempol+vehicle=$4.5 \pm 0.1$ versus $pD_2$ tempol+oxypurinol=$3.7 \pm 0.1$; $P < 0.05$) without significant changes in the maximum effect (Fig. 5A and C; $E_{max}$ tempol+vehicle=$101 \pm 1\%$ versus $E_{max}$ tempol+oxypurinol=$105 \pm 2\%$; $P > 0.05$).

We then compared the contribution of XOR to the vasodilatory effects of nitrite under control conditions (incubation with vehicle) with those found in the presence of the tempol (Fig. 6). The contribution of XOR to the effects of nitrite was assessed by calculating the decreases in $pD_2$ produced by oxypurinol ($\Delta pD_{2\text{ox}} = pD_2$ vehicle-$pD_2$ oxypurinol). Therefore, the comparison of $\Delta pD_{2\text{ox}}$ values obtained under control conditions with those obtained in the presence of the tempol could reflect how oxidative stress impairs XOR-mediated NO production and the corresponding vasodilatory effects of nitrite. We found that the $\Delta pD_{2\text{ox}}$ was more pronounced in the presence of tempol than in the absence of this drug (Fig. 6; $-0.81 \pm 0.11$ versus $-0.49 \pm 0.09$, respectively; $P < 0.05$), suggesting that antioxidant treatment protects NO formed by XOR by preventing NO-superoxide reaction, thus enhancing the vasodilatory effects of sodium nitrite in 2K1C hypertension.

To further examine relevant implications of the vascular findings above, we studied how XOR-mediated hypotensive responses
to nitrite (shown in Fig. 3) are modified by antioxidant treatment. In the presence of tempol, oxypurinol blunted the hypotensive responses to sodium nitrite, particularly when the highest doses (30 and 100 μmol/kg, Fig. 7A) were used. Then we compared the AUC of the changes in MAP induced by sodium nitrite in 2K1C hypertensive rats pretreated with tempol or vehicle, either in the presence or in the absence of oxypurinol (Fig. 7B). While tempol produced a small (not significant) improvement in the hypotensive responses to sodium nitrite in vehicle pretreated rats (Fig. 7B; P > 0.05) as previously reported [34], oxypurinol blunted nitrite-induced hypotension to a similar extent in the presence versus in the absence of tempol (Fig. 7B; P < 0.05), thus suggesting that oxidative stress does not affect the contribution of XOR to hypotensive responses to nitrite.

4. Discussion

This is the first study to show that vascular XOR contributes to the vasodilatory and hypotensive effects of sodium nitrite in renovascular hypertension. This finding is very important because it suggests that XOR inhibition prevents antihypertensive effects of nitrite in the 2K1C animal model of hypertension, which resembles human renovascular hypertension [22]. Another important finding is that tempol improves XOR-dependent vasodilatory effects of nitrite, suggesting a dual role for XOR in the pharmacology of nitrite. This result suggests that while XOR contributes to vasorelaxing effects of nitrite, its activity also contributes to nitroso-redox imbalance attenuating the vascular responses to nitrite-derived NO.

Nitrite used to be considered as a relatively inactive oxidation product of NO [17]. However, this view has changed dramatically in the last few decades, and nitrite is now regarded as an important source of NO independent of the classical enzymatic NO formation from l-arginine [13–16], and a candidate in the pharmacotherapy of cardiovascular diseases, especially hypertension. In fact, there is strong experimental evidence that nitrite lowers blood pressure in different animal models of hypertension including SHR [35], l-NAME [20,36,37], DOCA-salt [9] and 2K1C hypertension [32,37].

The mechanisms explaining the antihypertensive effects of nitrite remain poorly understood [38]. In this respect, XOR has been implicated in NO generation from nitrite [20,21], particularly in hypertension because XOR up-regulation has been shown in
different animal models of hypertension including SHR [8] and DOCA-salt [9,39]. However, few studies have reported contrasting results with respect to the participation of XOR in antihypertensive effects of nitrite [20,21]. While vascular XOR contributes to the acute hypotensive effects of nitrite in l-NAME hypertension [20], Ghosh et al. showed that erythrocytic XOR is more relevant to this effect, without the involvement of vascular XOR [21]. In the present study, we found increased vascular XOR activity and ROS levels in 2K1C hypertensive rats that may result of increased angiotensin II levels, a potent XOR activator [25,26]. Consistent with increased vascular XOR activity in hypertension, treatment with the XOR inhibitor oxypurinol impaired the vasodilatory responses of aortic rings from 2K1C rats to nitrite. These functional responses were further confirmed in vivo by assessing blood pressure responses. Interestingly, oxypurinol blunted the hypotensive responses to nitrite in 2K1C rats, particularly at the highest doses. Together, our findings suggest that increased vascular XOR activity improves the vascular and hypotensive responses to nitrite. If patients with clinical hypertension present pathophysiological alterations similar to those reported here, it is possible that the wide use of XOR inhibitors such as allopurinol [27] may cancel anti-hypertensive effects of nitrite. Clinical studies are needed to test this possibility.

In agreement with previous studies [5,31,33], we observed increased ROS levels in aortas from 2K1C rats compared with sham rats. While the main goal of the present study was not to detect a specific oxidant in 2K1C hypertension, and for this reason we used the term ROS in this manuscript [40], it is reasonable to suggest

Fig. 4. Evaluation of reactive oxygen species (ROS) levels in aortic rings from sham and 2K1C rats by dihydroethidium (DHE) fluorescence. Panel A shows representative photomicrographs (×400) with red fluorescence of DHE-aortic cryosections. Panel B shows the quantification of aortic DHE fluorescence in sham and 2K1C rats in the presence and the absence of tempol (10^-4 mol/l, a superoxide dismutase analogue). Panel C shows the quantification of aortic DHE fluorescence in positive control experiments using aortas from 2K1C rats in the presence of diphenyl iodonium (DPI 10 μmol/l, a flavoprotein inhibitor) and Tiron (1 mmol/l, a superoxide scavenger). Data are shown as mean ± S.E.M. (n=5–8/group). *P < 0.05 versus Sham and 2K1C + Tempol. #P < 0.05 versus 2K1C.
that increased XOR activity in hypertension enhances superoxide production, which readily inactivates nitrite-derived NO [28, 29], possibly impairing the responses to this NO metabolite. Therefore we examined whether the administration of tempol improves nitroso-redox balance of hypertension and enhances XOR-dependent vasodilatory and hypotensive effects of nitrite. Interestingly, treatment with tempol decreased vascular ROS levels and improved XOR-mediated vasodilatory responses to nitrite in 2K1C hypertension, thus suggesting that tempol improved nitrite-derived NO bioavailability and vasorelaxing responses. However, in contrast with the vascular responses to nitrite, antioxidant treatment was not associated with improved XOR-mediated hypotensive responses to nitrite. These results may reflect the idea hypotensive responses to nitrite may involve other mechanisms than the simple XOR-mediated generation of NO from nitrite. In addition, it is clear that blood pressure regulation involves a variety of determinants affecting cardiac output and total peripheral resistance, and therefore blood pressure responses are much more complex than vascular responses. Another possible explanation for the differential effects of tempol between the hypotensive responses and the aortic studies is that blood pressure responses in vivo were assessed in freely moving rats breathing room air (21% O₂), whereas aortic studies were carried out with 95% O₂ in the vessel bath. Under physiologic conditions (21% O₂) XOR reduces O₂ mostly to H₂O₂, whereas superoxide is the predominant product when O₂ concentrations increase to near 100% [41,42]. Therefore, at very high O₂ tension in the vessel bath, one would expect much greater superoxide formation from XOR than under in vivo conditions [41,42], and this artificial enhancement of superoxide levels could favor tempol to produce more observable effects.

In conclusion, our results show that XOR activity is very relevant to both vasorelaxing and antihypertensive responses to nitrite in renovascular hypertension, and the use of XOR inhibitors may cancel both effects. This finding suggests that nitrite treatment may not be effective in patients being treated with XOR inhibitors. However, clinical implications of these findings should be explored in future studies. Moreover, while tempol and other antioxidants may improve the vascular responses to nitrite, the antihypertensive responses are not affected.
Fig. 7. Antioxidant tempol does not enhance the contribution of xanthine oxidoreductase (XOR) to the hypotensive responses to sodium nitrite in 2K1C rats. Panel A shows the changes in mean arterial pressure (MAP) measured in unaesthetized free-moving 2K1C rats in response to increasing doses of sodium nitrite in the presence of the antioxidant tempol (18 mg/kg) plus the XOR inhibitor oxypurinol (25 mg/kg) or tempol (18 mg/kg) plus vehicle (NaOH, 0.15 mol/l). Panel B shows the area under the curve (AUC) of the changes in MAP induced by sodium nitrite in the presence or in the absence of tempol plus oxypurinol (or vehicle). Data are shown as mean ± S.E.M. (n=5–7 group). *P < 0.05 for Oxypurinol versus Vehicle.

Competing interests

A.N. Schechter: Use of nitrate salts for the treatment of cardiovascular conditions. United States Patents 20,060,182,815; 20,070,154,569; 20,100,247,682.

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