Sodium nitrite downregulates vascular NADPH oxidase and exerts antihypertensive effects in hypertension

Marcelo F. Montenegro a,⁎, Jefferson H. Amaral a, Lucas C. Pinheiro b, Eliana K. Sakamoto a, Grazielle C. Ferreira a, Rosana Inacio Reis c, Diogo M.O. Marçal a, Romaiana P. Pereira d, Jose E. Tanus-Santos a,⁎

a Department of Pharmacology, Faculties of Medicine of Ribeirao Preto, University of Sao Paulo, 14049–900 Ribeirao Preto, SP, Brazil
b Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas, 13081–970 Campinas, SP, Brazil
c Nephrology Division, Department of Medicine, Federal University of Sao Paulo, 04023–040 Sao Paulo, SP, Brazil
d Department of Chemistry, Federal University of Santa Maria, 97105–900 Santa Maria, RS, Brazil

⁎ Corresponding authors. Fax: + 55 16 3633 2301.
E-mail addresses: marcelofm@pq.cnpq.br (M.F. Montenegro), tanus@fmrp.usp.br, tanussantos@yahoo.com (J.E. Tanus-Santos).

Original Contribution

Dietary nitrite and nitrate are important sources of nitric oxide (NO). However, the use of nitrite as an antihypertensive drug may be limited by increased oxidative stress associated with hypertension. We evaluated the antihypertensive effects of sodium nitrite given in drinking water for 4 weeks in two-kidney one-clip (2K1C) hypertensive rats and the effects induced by nitrite on NO bioavailability and oxidative stress. We found that, even under the increased oxidative stress conditions present in 2K1C hypertension, nitrite reduced systolic blood pressure in a dose-dependent manner. Whereas treatment with nitrite did not significantly change plasma nitrite concentrations in 2K1C rats, it increased plasma nitrate levels significantly. Surprisingly, nitrite treatment exerted antioxidant effects in both hypertensive and sham-normotensive control rats. A series of in vitro experiments was carried out to show that the antioxidant effects induced by nitrite do not involve direct antioxidant effects or xanthine oxidase activity inhibition. Conversely, nitrite decreased vascular NADPH oxidase activity. Taken together, our results show for the first time that nitrite has antihypertensive effects in 2K1C hypertensive rats, which may be due to its antioxidant properties resulting from vascular NADPH oxidase activity inhibition.

© 2011 Elsevier Inc. Open access under the Elsevier OA license.

0891-5849 © 2011 Elsevier Inc. Open access under the Elsevier OA license.
markers of NO bioavailability. Together, our results show for the first time that nitrite has antihypertensive properties in 2K1C hypertensive rats. Our findings support the new idea that nitrite exerts antioxidant effects by downregulating vascular NADPH oxidase activity.

Materials and methods

Animals and treatments

This study complied with guidelines of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, and the animals were handled according to the guiding principles published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Wistar rats (180–200 g) obtained from the colony at the University of São Paulo (Ribeirão Preto Campus, Brazil) were maintained on a 12-h light/dark cycle at room temperature (22–25 °C) with free access to standard rat chow and water. 2K1C hypertension was induced by clipping the left renal artery with a silver clip (0.2 mm) [33]. Sham-operated rats underwent the same surgical procedure except for the clip placement. Ketamine (100 mg/kg) and xylazine (10 mg/kg) were used ip to anesthetize the animals. Treatment with sodium nitrite (or vehicle) was started 2 weeks after surgery and maintained for 4 weeks. The sham-operated rats were randomly divided into four groups (n = 12/group) and were treated for 4 weeks with vehicle or increasing concentrations (0.5, 5, and 50 mM) of sodium nitrite in their drinking water (sham + vehicle, sham + 0.5 mM, sham + 5 mM, and sham + 50 mM). Similarly, the 2K1C rats were randomly divided into four groups (n = 12/group) and also were treated for 4 weeks with vehicle or increasing concentrations (0.5, 5, and 50 mM) of sodium nitrite in their drinking water (2K1C + vehicle, 2K1C + 0.5 mM, 2K1C + 5 mM, and 2K1C + 50 mM). The drinking water consumption was monitored daily to estimate the nitrite ingested and the solutions were changed every 2 days to avoid nitrate formation. The calculated mean daily nitrite ingestion resulted in approximately 5.6, 61, and 85 mg/kg of nitrite in the groups treated with 0.5, 5, and 50 mM nitrite in their drinking water, respectively.

Body weight and tail systolic blood pressure (SBP) were assessed weekly by tail-cuff plethysmography. To minimize the effects of stress induced by this method on blood pressure measurement, the animals were trained for a week before surgery.

Measurement of plasma NOx (nitrate + nitrite) concentrations

Arterial blood samples were collected in tubes containing heparin and immediately centrifuged at 1000 g for 3 min. Plasma aliquots were stored at −70 °C until analyzed. The plasma NOx concentrations were determined in duplicate by using the Griess reaction as we described previously [34,35]. Briefly, 40 μl of plasma was incubated with the same volume of nitrate reductase buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM β-nicotinamide adenine dinucleotide phosphate, and 2 U/ml nitrate reductase) in individual wells of a 96-well plate. Samples were allowed to incubate overnight at 37 °C in the dark. Eighty microliters of freshly prepared Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) was added to each well and the plate was incubated for an additional 15 min at room temperature. A standard nitrate curve was obtained by incubating sodium nitrate (0.2–200 μM) with the same reductase buffer. We found a strong correlation between the standard curve measurements of nitrate and nitrite (R² = 0.999), and the recovery of nitrate was ~98% (data not shown). The absorbances were measured at 540 nm using a microplate reader. The assay results were not significantly affected by the presence of plasma proteins.

Measurement of plasma nitrite concentrations

Plasma aliquots were analyzed in duplicate for their nitrite content using an ozone-based reductive chemiluminescence assay as previously described [35,36]. Briefly, to measure nitrite concentrations in plasma, 300 μl of plasma sample was injected into a solution of acidified tri-iodide, purging with nitrogen in-line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO analyzer, Boulder, CO, USA). Approximately 8 ml of tri-iodide solution (2 g potassium iodide and 1.3 g iodine dissolved in 40 ml water with 140 ml acetic acid) was placed in the purge vessel into which plasma samples were injected. The tri-iodide solution reduces nitrites to NO gas, which is detected by the NO analyzer. The data were analyzed using the software Origin Lab 6.1.

Assessment of lipid peroxide and 8-isoprostane levels in plasma

Plasma lipid peroxide levels were determined by measuring thiobarbituric acid-reactive substances (TBARS) using a fluorimetric method as previously described [34]. This method requires excitation at 515 nm and emission at 535 nm and uses 1,1,3,3-tetramethoxypropane as standard [37]. The lipoperoxide levels were expressed in terms of malondialdehyde (nmol/ml).

To further study oxidative stress, plasma 8-isoprostanes (8-isoPGF2α) concentrations were measured with commercially available enzyme-linked immunosorbent assay kits (Cayman Chemical Co., Ann Arbor, MI, USA), according to the manufacturer’s instructions.

Measurement of vascular reactive oxygen species (ROS) production

Superoxide production in the aorta was measured by dihydroethidium (DHE), a ROS-sensitive fluorescent dye. The aortic cryosections (5 μm thick) were incubated at room temperature with DHE (10 μmol/L) for 30 min. Sections were examined by fluorescence microscopy (Leica Imaging Systems Ltd., Cambridge, UK) and the image was captured at ×400. Red fluorescence from 20 fields around the vessel was evaluated using ImageJ software (http://rsbweb.nih.gov/ij/), as described before [33]. Some experiments were performed in the absence or presence of tiron (1 mmol/L), an O2•− scavenger; diphenyliodonium (DPI; 10 μmol/L), an inhibitor of flavoproteins; apocynin (1 mmol/L), a nonselective inhibitor of NADPH oxidase; or increasing concentration of nitrite (0.1, 1, and 10 μM). The readings were performed after preincubation for 20 min with the inhibitors or nitrite.

Evaluation of possible antioxidant effects of nitrite

To assess possible antioxidant properties of nitrite, we evaluated the O2•− scavenging capacity of nitrite by using pyrogallol as a source of O2•− and lucigenin chemiluminescence. Briefly, 50 μl of pyrogallol (24 mM in 10 mM HCl) was added to 900 μl of Tris–HCl buffer (50 mM, pH 8.2) containing 5 μM lucigenin. Fifty microliters of vehicle, ascorbic acid (100 μmol/L), or increasing concentrations of nitrite (0.1 to 100 μM) were tested using a chemiluminescence reader (Berthold FB12 single-tube lumimeter) at 37 °C.

In addition, we used absorption spectroscopy to assess whether pyrogallol autoxidation is decreased by nitrite. Pyrogallol spontaneously autoxidizes, thus generating O2•−, which can be inhibited by superoxide dismutase or antioxidants. The rate of pyrogallol autoxidation is measured spectrophotometrically at 420 nm [38]. In brief, we mixed in a cuvette 100 μl of vehicle or ascorbic acid (100 μM) or nitrite (1 to 100 μM), 150 μl of pyrogallol (24 mM in 10 mM HCl), and 2750 μl of Tris–HCl buffer (50 mM, pH 8.2). The kinetics readings at 420 nm began immediately and lasted for 7 min at 25 °C. A similar approach was used to evaluate the possibility that nitrite decreases epinephrine autoxidation, which also generates O2•− that can be scavenged by antioxidants. Briefly, 100 μl of vehicle, ascorbic acid (100 μM), or nitrite (1 to 100 μM)
was mixed with 2.8 ml of 50 mM sodium carbonate buffer, pH 10.2, containing 0.1 mM EDTA. The reaction was then initiated by adding 100 μl of epinephrine (10 mM prepared in 10 mM HCl) and the kinetics readings were performed at 480 nm, as described before [39].

In vitro effects of nitrite on lipid peroxidation

We evaluated whether nitrite affects lipid peroxidation. In brief, under deep urethane anesthesia (1.2 g/kg), untreated rats were euthanized and their livers were collected and placed on ice. Tissues were immediately homogenized in cold 10 mM Tris–HCl, pH 7.5 (1/10, w/v), and the homogenates were centrifuged for 15 min at 3000 rpm. The supernatants (100 μl) were mixed with freshly prepared FeSO₄ (10 μM), in the absence or in the presence of vehicle or increasing concentrations of nitrite (1, 10, and 100 μM) or quercetin (100 μM) and then incubated for 1 h at 37 °C. Some experiments were performed without FeSO₄ as controls (basal), and after the incubation period, the amount of thiobarbituric acid-reactive substances formed was determined as previously described [40]. The rationale for these experiments is that iron increases lipid peroxidation in the tissue homogenates, which can be prevented by antioxidants [41].

Assessment of plasma xanthine oxidase activity

Plasma xanthine oxidase activities were measured with a commercial kit (Amplex red xanthine/xanthine oxidase assay kit; Molecular Probes, Eugene, OR, USA), following the manufacturer's instructions.

Western blotting analysis for xanthine oxidase

To evaluate the vascular expression of xanthine oxidase, Western blotting was performed as described previously [42], with primary antibodies (1:500) directed against xanthine oxidase (sc-20991; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the expression was corrected to β-actin expression (dilution 1:10.000, MAB1501; Millipore, Temecula, CA, USA).

Measurement of NADPH oxidase activity

NADPH-dependent superoxide production was measured in aortic rings from all experimental groups. Aortic rings were transferred to luminomicrotubes containing 1 ml of Hanks’ buffer, pH 7.2. After equilibration and background counts, a non-redox-cycling concentration of lucigenin (5 μmol/L) and β-NADPH (12 μmol) was automatically added and the luminescence counts were measured continuously for 15 min in a Berthold FB12 single-tube luminometer at 37 °C. Background signals from the aortic rings were subtracted from the β-NADPH-driven signals and the results were normalized for the dry weight and reported as lucigenin chemiluminescence/mg of dry tissue, as described previously [43]. Some experiments were performed in the absence or presence of PEG–SOD (200 U/ml), a cell-permeative polyethylene glycol–superoxide dismutase; DPI (10 μmol/L), an inhibitor of flavoproteins; apocynin (1 mmol/L), a nonselective inhibitor of NADPH oxidase; allopurinol (10 μmol/L), a xanthine oxidase inhibitor; or rotenone (10 μmol/L), a mitochondrial electron transport inhibitor.

Drugs and solutions

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

Statistical analysis

The results are expressed as means ± SEM. The comparisons between groups were assessed by two-way analysis of variance using Bonferroni correction or one-way analysis of variance followed by Dunnett’s multiple comparison tests. A probability value < 0.05 was considered significant.

Results

Treatment with sodium nitrite ameliorates 2K1C hypertension in a dose-dependent manner

Baseline SBP and body weight were similar in all experimental groups (Figs. 1A and B). Whereas no significant changes in SBP were seen in the sham- and sham-nitrite-treated groups, SBP increased in the 2K1C groups after the first week of the renal artery surgery and peaked at week 4 in the 2K1C + vehicle group (Fig. 1A). Conversely, treatment of the 2K1C rats with nitrite induced significant decreases in SBP in all 2K1C-nitrite-treated groups in a dose-dependent manner (Fig. 1A; P < 0.05).

We found no significant differences in body weight when all the experimental groups were compared (Fig. 1B, P = 0.05), except for both sham and 2K1C groups receiving 50 mM nitrite, which gained less weight than the other groups (Fig. 1B, P < 0.05). Interestingly, the average drinking water consumption was 68 ± 11 ml/rat/day in groups receiving only water or nitrate at 0.5 and 5 mM in the drinking water. However, we found a 37% lower consumption in the groups of rats receiving nitrate at 50 mM.

Sodium nitrite treatment restored plasma NOx levels in 2K1C hypertension

We examined whether sodium nitrite could reverse the lower NO availability in 2K1C hypertension. Significantly lower plasma NOx and nitrite levels were found in the 2K1C + vehicle group compared with those found in the sham + vehicle group (P < 0.05; Figs. 2A and B, respectively). Interestingly, nitrite treatment induced no significant increases in plasma nitrite concentrations in either the sham- or the 2K1C-nitrite-treated group (P > 0.05; Fig. 2B), although the higher dose tended to increase plasma nitrite levels in the 2K1C + 50 mM group. Conversely, nitrite treatment induced a dose-dependent increase in the plasma NOx levels in 2K1C hypertensive rats, as shown in Fig. 2A (P < 0.05). However, significant increases in plasma NOx were found in sham-nitrite-treated rats only with the higher dose (50 mM; P < 0.05; Fig. 2A).

Sodium nitrite treatment produces relevant antioxidant effects

To evaluate the effects of hypertension and nitrite treatment on oxidative stress, we used three assays, with two of them assessing oxidative stress, we used three assays, with two of them assessing oxidative stress, we used three assays, with two of them assessing oxidative stress. We assessed the lipid peroxide levels by measuring thiobarbituric acid-reactive substances, and the results showed increased thiobarbituric acid-reactive substances (expressed in terms of malondialdehyde; MDA) in the 2K1C + vehicle group compared with the sham + vehicle group (P < 0.01; Fig. 3A). Interestingly, treatment with sodium nitrite produced antioxidant effects, as suggested by the significantly lower lipid peroxide levels in both sham- and 2K1C-nitrite-treated rats compared with their respective controls (sham + vehicle and 2K1C + vehicle group, respectively; P < 0.01; Fig. 3A). We confirmed these findings by assessing the plasma levels of 8-isoprostanes. In parallel with thiobarbituric acid-reactive substances, we found higher plasma 8-isoprostane levels in the 2K1C-vehicle-treated hypertensive rats compared with sham-vehicle-treated rats (P < 0.01; Fig. 3B). In the sham groups, nitrite treatment reduced 8-isoprostane levels when used at 0.5 and 50 mM (P < 0.05; Fig. 3B), but not at 5 mM concentration (P > 0.05;
Fig. 3B. In addition, nitrite treatment reduced 8-isoprostane levels when given at 0.5, 5, or 50 mM concentrations to 2K1C hypertensive rats ($P_{b} < 0.05$; Fig. 3B).

Nitrite treatment decreases the production of vascular ROS in 2K1C hypertension

To confirm the effects of hypertension and nitrite treatment on oxidative stress, we used a third assay. We evaluated ROS production by using the sensitive probe DHE in aortic slices from the animals. We found that DHE oxidation was significantly increased in the 2K1C vehicle group compared to the sham groups ($P < 0.05$, Figs. 4A and B). Treatment of 2K1C rats with nitrite attenuated 2K1C-induced oxidative stress in a dose-dependent manner ($P < 0.05$, Figs. 4A and B), and no effects were found in the sham-nitrite-treated groups.

To examine whether nitrite itself could directly decrease aortic DHE oxidation, we did a series of independent experiments in which we preincubated aortic slices of 2K1C hypertensive rats with increasing concentrations of nitrite. Preincubation of aortic slices with nitrite (0, 0.1, 1, or 10 μmol/L) for 30 min had no significant effects on DHE oxidation ($P > 0.05$, Fig. 4C). Conversely, preincubation of aortic slices with tiron (1 mmol/L), a powerful direct $O_2^- \cdot$ scavenger,
Nitrite does not have direct antioxidant properties

To examine whether nitrite could exert direct antioxidant effects, we conducted a series of in vitro experiments addressing any possible $O_2^-$ scavenging property of nitrite. Fig. 5A shows that nitrite was unable to scavange $O_2^-$ generated from pyrogallol, as assessed by lucigenin chemiluminescence, even when nitrite was tested at 100 μmol/L (P > 0.05). Conversely, ascorbic acid was tested as a positive control and it prevented the increases in lucigenin chemiluminescence induced by pyrogallol (P < 0.05; Fig. 5A).

Further confirming these findings, nitrite had no effect on iron-induced increases in lipid peroxidation in liver samples (P > 0.05; Fig. 5B), whereas quercetin, an antioxidant flavonoid used here as a positive control, blunted such effects (P < 0.05; Fig. 5B).

In addition to these findings, nitrite was unable to decrease pyrogallol and epinephrine autoxidation (P > 0.05; Figs. 5C and D, respectively), which are processes highly dependent on $O_2^-$ generation. Again, ascorbic acid was used as a positive control and it prevented both pyrogallol and epinephrine autoxidation (P < 0.05; Figs. 5C and D, respectively).

Sodium nitrite had small effects on plasma xanthine oxidase activity and no effects on aortic xanthine oxidase expression

We evaluated plasma xanthine oxidase activity in both sham normotensive and 2K1C hypertensive rats. As expected, we found increased plasma xanthine oxidase activity in the 2K1C vehicle group compared with the sham vehicle group (P < 0.05; Fig. 6A). Whereas treatment with nitrite induced no changes in xanthine oxidase activity in the sham groups, we found a small decrease in xanthine oxidase activity in the 2K1C nitrite 50 mM group (P < 0.05; Fig. 6A).

The analysis of xanthine oxidase expression in the aortas showed a trend for increased xanthine oxidase expression in 2K1C hypertensive rats compared with sham animals (P < 0.05; Fig. 6B) and no significant effects for nitrite.

Nitrite treatment reduces the NADPH oxidase activity in 2K1C hypertension

Because our results showed that nitrite was unable to exert direct $O_2^-$-scavenging properties, although it decreased lipid peroxidation and decreased aortic superoxide levels in 2K1C rats, with only minor effects on xanthine oxidase activity, we decided to study vascular NADPH oxidase activity because this is an important pro-oxidant enzyme, especially in hypertension. We measured NADPH oxidase activity in aortas from all experimental groups using lucigenin chemiluminescence. Surprisingly, treatment with sodium nitrite significantly decreased NADPH oxidase activity in both sham- and 2K1C-nitrite-treated rats (P < 0.01; Fig. 6A). In a series of control experiments, we showed that the chemiluminescence signal was suppressed by PEG–SOD and by the nonselective NADPH oxidase inhibitors DPI and apocynin, thus indicating that the signal was not an artifact (P < 0.01; Fig. 6B). Finally, the pretreatment of aortic rings with allopurinol or rotenone had no effect on NADPH-induced superoxide production (P > 0.05; Fig. 6B), thus suggesting that xanthine oxidase and mitochondria are not relevant contributors to superoxide production.

Discussion

The main findings of this study were: (i) nitrite treatment reduced SBP of 2K1C hypertensive rats; (ii) nitrite treatment exerts antioxidant effects; and (iii) although nitrite does not have direct antioxidant properties, it downregulates vascular NADPH oxidase activity. These data suggest that, despite increased oxidative stress present in hypertension, nitrite exerts relevant antihypertensive effects. These findings set the stage for the idea that overall vascular effects associated with nitrite may involve antioxidant mechanisms in addition to its bioconversion to NO.

Several reports have described vasodilator effects produced by nitrite, which are ascribed to nitrite oxide formation [8]. However, the effect of nitrite in hypertension has been poorly studied. The first study addressing possible antihypertensive effects of nitrite was reported by Classen et al. [26]. They found that a single oral dose of nitrite acutely decreased blood pressure in spontaneously hypertensive rats (SHR) [26]. Later, the same group showed that nitrite attenuated further increases in SBP found in SHR [44, 45]. Interestingly, nitrite did not decrease blood pressure in these studies, although it prevented further increases in blood pressure found in matched controls treated with water [45]. In parallel with these findings, Tsuchiya et al. showed that nitrite attenuated the increases in blood pressure caused by LNAME, an inhibitor of NO synthases [46]. In the present study, treatment with nitrite was started 2 weeks after hypertension was induced, so that we would make sure that the animals were really hypertensive when nitrite treatment was started. Interestingly, we found significant antihypertensive effects after the first week of treatment, and this effect improved with time. To our knowledge, this is the first study clearly showing antihypertensive effects exerted by nitrite in 2K1C hypertensive rats.

We found lower plasma nitrite and NOx levels in the 2K1C+ vehicle group compared with the sham+ vehicle group, thus suggesting that hypertension decreases NO availability. Treatment with nitrite did not
increased nitrite levels in the sham or in the 2K1C nitrite groups, despite a trend for increased nitrite levels with the higher nitrite dose in the 2K1C+nitrite 50 mM group. Conversely, whereas no changes were seen in plasma NO\textsubscript{x} levels in the sham groups, treatment with nitrite induced dose-dependent increases in plasma NO\textsubscript{x} levels in hypertensive rats. Together, these findings suggest that nitrite is continuously converted to nitrate and that supplying more nitrite results only in its oxidation to nitrate. Indeed, most plasma NO\textsubscript{x} levels correspond to nitrate [6,43], and the high concentrations of oxyhemoglobin usually found in the bloodstream can easily convert nitrite to nitrate [47,48].

The differences in plasma NO\textsubscript{x} levels found between normotensive and hypertensive rats reported here suggest that the kinetic profiles of nitrite/nitrate are different in normotensive versus hypertensive animals. Further studies assessing nitrate excretion in urine from normotensive and hypertensive animals are needed. The increases in plasma NO\textsubscript{x} levels found in hypertensive rats after nitrite treatment, but not in normotensive rats, may be explained by the fact that 2K1C hypertensive rats have impaired renal function and therefore lower renal nitrate clearance, whereas normotensive rats do not have this alteration.

Our findings suggest that nitrite may offer some advantages in the treatment of hypertension compared with other NO-donor drugs, especially in a context of increased oxidative stress. NO donors may have toxic effects resulting from the reaction between superoxide anion and NO released by these drugs, thus forming peroxynitrite [49]. Our results show that nitrite decreases blood pressure, even in a context of increased oxidative stress. Indeed, we demonstrated that nitrite reduced lipid peroxide levels in both sham and 2K1C rats, thus suggesting antioxidant effects produced by this compound. Further supporting this idea, we evaluated in situ ROS production in the aortas.

\[\text{Fig. 4. Effects of nitrite treatment on in situ vascular } \text{O}_2^- \text{ production measured by DHE fluorescence. (A) The fluorescence intensity in each experimental group. (B) Representative photomicrographs (original magnification \times 400) of arteries incubated in the presence of DHE, which produces a red fluorescence when oxidized to hydroxyethidium by } \text{O}_2^- \text{. (C) The effects induced by pretreatment of aortic slices (for 20 min) with vehicle or increasing concentrations of nitrite on DHE oxidation. (D) Results for positive controls. This graph shows reduced DHE oxidation in 2K1C aortic slices previously treated for 20 min with a nonselective NADPH oxidase inhibitor, apocynin or DPI, as well as with the } \text{O}_2^- \text{ scavenger tiron. Data are shown as means } \pm \text{ SEM (n = 5 per group). *P < 0.05 versus sham vehicle group. #P < 0.05 versus 2K1C vehicle group.} \]
from studied animals. By using the ROS-sensitive probe DHE, we found that treatment with nitrite decreased aortic DHE oxidation in 2K1C rats, thus confirming antioxidant properties of nitrite treatment. Interestingly, nitrite itself had no direct effects on the increased aortic DHE oxidation found in hypertensive rats, as suggested by the lack of any effects of nitrite in experiments in which we preincubated aortic slices from sham and 2K1C hypertensive rats with increasing concentrations of nitrite. Conversely, preincubation of aortas with tiron, a powerful direct \( \text{O}_2^- \) scavenger, significantly reduced DHE oxidation. Similar results were found when we used the nonselective NADPH oxidase inhibitors apocynin and DPI. Together, these findings are consistent with antioxidant effects exerted by nitrite.

To show further evidence that nitrite does not exert direct antioxidant effects, we used different in vitro experimental approaches to examine whether nitrite could have any \( \text{O}_2^- \)-scavenging capacity. Nevertheless, nitrite (even at 100 \( \mu \)M concentration) was unable to show any antioxidant effects. Moreover, nitrite had no direct effects on lipid peroxidation. Overall, our results provide strong evidence ruling out the possibility that nitrite could have direct antioxidant effects.

We evaluated whether treatment with nitrite could inhibit xanthine oxidase or NADPH oxidase and maybe explain the antioxidant effects reported above. Both enzymes have been implicated as important pro-oxidant enzymes contributing to hypertension [32, 50, 51]. As expected, we found increased xanthine oxidase activity in 2K1C hypertensive rats treated with vehicle, and treatment with nitrite induced a small decrease in xanthine oxidase activity in the 2K1C hypertensive rats treated with the highest nitrite dose. Importantly, xanthine oxidase can synthesize nitric oxide from nitrite. If xanthine oxidase promotes nitric oxide formation from nitrite, it is possible that the increases in xanthine oxidase activity in hypertension that we and others have reported [42] can exert beneficial effects rather than it being a deleterious pro-oxidant enzyme. However, this hypothesis remains to be tested.

In parallel with xanthine oxidase activity, we found increased NADPH oxidase activity in 2K1C hypertensive rats treated with vehicle. However, treatment with nitrite significantly decreased vascular NADPH oxidase activity in both sham- and 2K1C-nitrite-treated rats. These results, together with the lower lipid peroxide levels and decreased aortic DHE oxidation in 2K1C rats treated with nitrite, are
consistent with the suggestion that nitrite exerts relevant antioxidant effects by inhibiting vascular NADPH activity. Interestingly, we have previously shown antioxidant effects exerted by nitrite in an animal model of acute pulmonary thromboembolism [21,23]. Nitrite infusions attenuated the pulmonary hypertension caused by acute pulmonary thromboembolism and this effect was associated with lower lipid peroxidation [21,23]. Most importantly, nitrite reduced the production of superoxide by neutrophils stimulated with phorbol 12-myristate 13-acetate, an NADPH oxidase activator [21]. Finally, our findings underscore the notion that, in addition to its vasodilator properties, nitrite may have relevant antioxidant effects that contribute to its antihypertensive effects reported here.

Some limitations of our study should be taken into consideration. First, caution should be taken in interpreting the plasma activity of xanthine oxidase. Although increased xanthine oxidase expression has been reported in hypertension [42], we found only a trend for increased expression. However, the increased xanthine oxidase activity in vascular tissues could help to explain the effects exerted by nitrite in this study. Second, in this study, we were unable of define precise mechanisms explaining how nitrite inhibits NADPH-induced superoxide production. The molecular mechanism, as well as which NADPH oxidase isoform is inhibited by nitrite, remains unclear and is currently under investigation. Third, caution should be taken with respect to the NADPH inhibitors used here (DPI and apocynin), which are nonselective. Apocynin may have antioxidant effects rather than being an NADPH oxidase inhibitor [52]. Finally, it might be relevant to study methemoglobin formation as a result of nitrite treatment, particularly when nitrite is used at high doses (50 mM), possibly explaining why the animals gained less weight with this dose.

In conclusion, our results show evidence that nitrite has antioxidant effects, probably explained by NADPH oxidase downregulation. Our findings show a new and relevant mechanism for the antihypertensive properties of nitrite in 2K1C hypertensive rats.

Acknowledgments

This study was funded by the Fundação de Amparo a Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico, and Programa Nacional de Cooperação Acadêmica.

Fig. 6. Activity and expression of xanthine oxidase in all experimental groups. (A) Plasma xanthine oxidase activity. (B) A representative Western blotting gel showing the expression of xanthine oxidase in the aortas from rats and a bar graph showing the densitometric data. β-Actin content was used for normalization. Data are shown as means ± SEM (n = 10–12 per group for activity and n = 6 per group for Western blotting results). *P < 0.05 versus the sham vehicle group. #P < 0.05 versus the 2K1C vehicle group.

Fig. 7. Nitrite decreases vascular NADPH oxidase activity. (A) NADPH oxidase activity in the aortas from all experimental groups. (B) The chemiluminescence signal is almost abolished when the aortic rings are incubated for 30 min with a nonselective NADPH oxidase inhibitor, DPI (2K1C + DPI) or apocynin (2K1C + Apocynin), as well as with the cell-permeative polyethylene glycol–superoxide dismutase (2K1C + PEG–SOD). However, the xanthine oxidase inhibitor allopurinol (2K1C + Allopurinol) or the inhibitor of mitochondrial electron transport rotenone (2K1C + Rotenone) had no effects. Data are shown as means ± SEM (n = 10 per group). *P < 0.05 versus the sham vehicle group. #P < 0.05 versus the 2K1C vehicle group. n.s., nonsignificant.
References


