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## Research Paper

## Consistent antioxidant and antihypertensive effects of oral sodium nitrite in DOCA-salt hypertension

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## ABSTRACT

Hypertension is a common disease that includes oxidative stress as a major feature, and oxidative stress impairs physiological nitric oxide (NO) activity promoting cardiovascular pathophysiological mechanisms. While inorganic nitrite and nitrate are now recognized as relevant sources of NO after their bioactivation by enzymatic and non-enzymatic pathways, thus lowering blood pressure, mounting evidence suggests that sodium nitrite also exerts antioxidant effects. Here we show for the first time that sodium nitrite exerts consistent systemic and vascular antioxidant and antihypertensive effects in the deoxycorticosterone-salt (DOCA-salt) hypertension model. This is particularly important because increased oxidative stress plays a major role in the DOCA-salt hypertension model, which is less dependent on activation of the renin-angiotensin system than other hypertension models. Indeed, antihypertensive effects of oral nitrite were associated with increased plasma nitrite and nitrate concentrations, and completely blunted hypertension-induced increases in plasma 8-isoprostane and lipid peroxide levels, in vascular reactive oxygen species, in vascular NADPH oxidase activity, and in vascular xanthine oxidoreductase activity. Together, these findings provide evidence that the oral administration of sodium nitrite consistently decreases the blood pressure in association with major antioxidant effects in experimental hypertension.

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## Introduction

Nitrate and nitrite ( $\text{NO}_3^-/\text{NO}_2^-$ ) are not considered as simple products of nitric oxide (NO) metabolism anymore because they recycle back to NO under certain conditions via a new biological nitrate–nitrite–NO pathway [1]. This pathway complements the classic enzymatic NO formation from L-arginine by NO synthase (NOS), especially when NOS-derived NO formation is impaired such as during hypoxia [1]. In addition, nitrate can be bioactivated after orally-ingested nitrate enters the enterosalivary circuit to generate inorganic nitrite in the mouth [2,3]. Oral nitrite is then swallowed and reduced to NO under the acidic conditions of the stomach [3,4], which are critical to the antihypertensive effects of oral nitrite [5,6]. However, mounting evidence now suggests that a variety of other enzymes with nitrite reductase activity can also systemically generate NO from nitrite [7–13] and contribute to its antihypertensive effects. On the other hand, nitrite signaling may activate other relevant pathways [14] resulting in

antihypertensive effects, such as pathways that result in antioxidant effects and explain at least part of the antihypertensive effects of this anion [15,16].

A major feature of hypertensive disorders includes oxidative stress [17], which impairs NO bioavailability and is associated with lower nitrite levels as compared to normotensive controls [18–20]. While antihypertensive effects of sodium nitrite have been demonstrated in some animal models of hypertension [11,15,16,19–24], no previous study has addressed the possible antihypertensive effects of oral sodium nitrite in the deoxycorticosterone-salt (DOCA-salt) hypertension model. This is particularly important because increased oxidative stress plays a major role in this model of hypertension [25–27], and it also provides important information regarding the dependence of the antihypertensive effects on interference with the renin-angiotensin system (RAS). This is because, in contrast with the two-kidney, one-clip model [28,29], the DOCA-salt model resembles the clinical condition of abnormal aldosterone secretion, and therefore is less dependent on activation of the RAS, which is apparently not affected by therapy with oral nitrite, as recently reported [21].

Given the major role of oxidative stress in the DOCA-salt hypertension model [25,27,30] and the possible antioxidant effects of sodium nitrite [15,16,31], this study aimed at examining

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antioxidant and antihypertensive effects of sodium nitrite in this hypertension model. Consistent with previous findings, our results show that oral sodium nitrite exerts antioxidant effects that result in lower blood pressure in this hypertension model.

## Materials and methods

### Animals and treatments

This study followed the guidelines of the Faculty of Medicine of Ribeirão Preto of the University of São Paulo, and the rats were handled according to the guiding principles published by 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 University of São Paulo (Ribeirão Preto Campus) were maintained at room temperature (22–25 °C) on a 12-h light/dark cycle with free access to standard rat chow and water. DOCA-salt hypertension [25,27,30] was induced after the rats underwent uninephrectomy under tribromoethanol (250 mg/kg i.p.) anesthesia. One week after surgery, the rats received subcutaneous injections of deoxycorticosterone (DOCA; 30 mg/kg/week), and their drinking water was supplemented with 1.0% NaCl and 0.2% KCl. Control rats received vehicle injections and normal tap drinking water.

The rats were randomly divided into 4 experimental groups ( $n=10$ /group): Unx+vehicle (uninephrectomized animals who received tap water and daily gavage of vehicle), Unx+nitrite (uninephrectomized animals who received tap water and daily gavage of nitrite), DOCA+vehicle (uninephrectomized animals who received subcutaneous injection of DOCA weekly and daily gavage of vehicle), and DOCA+nitrite (uninephrectomized animals who received subcutaneous injection of DOCA weekly and daily gavage of nitrite).

After 2 weeks of treatment of uninephrectomized rats with tap water (control groups) or DOCA (hypertensive groups), the animals received a daily gavage of vehicle or sodium nitrite (15 mg/kg) for four additional weeks. This dose of sodium nitrite was chosen with basis on previous studies showing that it exerts relevant antihypertensive effects [21]. 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 the protocol started.

### Measurement of plasma nitrite concentrations

Six hours after the last nitrite gavage, the rats were anesthetized with tribromoethanol (250 mg/kg), and arterial blood samples were collected into tubes containing heparin and immediately centrifuged at 1000g for 3 min. Plasma aliquots were stored at  $-70$  °C until analyzed. Plasma aliquots were analyzed in duplicate for their nitrite content using an ozone-based reductive chemiluminescence assay as previously described [32]. Briefly, to assess nitrite content in plasma, 200  $\mu$ l of plasma samples were injected into a solution of acidified tri-iodide, purged 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 8.5.

### Measurement of plasma NO<sub>x</sub> (nitrate+nitrite) concentrations

The plasma NO<sub>x</sub> concentrations were determined in duplicate by using the Griess reaction as previously described [33]. Briefly, 40  $\mu$ l of plasma were incubated with the same volume of nitrate reductase buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM beta nicotinamide adenine dinucleotide phosphate, and 2 units of nitrate reductase/ml) in individual wells of 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) were 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  $\mu$ M) with the same reductase buffer. The absorbance was measured at 540 nm, using a microplate reader.

### Assessment of plasma 8-isoprostane and lipid peroxide levels

Plasma samples from all groups were used to measure 8-isoprostanes (8-isoPGF<sub>2</sub> $\alpha$ ) concentrations. The measurements were carried out as previously described [34], with commercially available enzyme-linked immunosorbent assay kits (Cayman Chemical Co., Ann Arbor, MI, USA).

The plasma levels of lipid peroxide were determined by measuring thiobarbituric acid reactive substances (TBARS) using a fluorimetric method (with excitation at 515 nm and emission at 553 nm) as detailed previously [35]. The lipoperoxide levels were expressed in terms of malondialdehyde (MDA) (nmol/ml).

### Measurement of vascular reactive oxygen species (ROS) production

To assess vascular oxidative stress, ROS production in the aorta from animals was measured by dihydroethidium (DHE), a ROS-sensitive fluorescent dye, as previously described [36]. Aortic cryosections (5  $\mu$ m thick) were incubated at room temperature with DHE (10  $\mu$ mol/l) for 30 min. Sections were examined by fluorescence microscopy (Leica Imaging Systems Ltd., Cambridge, UK) and the image was captured at X400. Red fluorescence from 20 fields around the vessels was evaluated using ImageJ software (<http://rsbweb.nih.gov/ij/>).

### Assessment of vascular xanthine oxidoreductase activity

Aortic xanthine oxidoreductase activity was measured as previously described [11], with a commercial kit (Amplex red xanthine/xanthine oxidase assay kit; Molecular Probes, Eugene, OR, USA), following the manufacturer's instructions.

### Measurement of vascular NADPH-dependent ROS production

NADPH-dependent ROS production was measured in aortic rings from rats ( $n=6-8$ /group) as previously described [15]. Aortic rings were transferred to luminescence vials containing 1 ml of Hanks' buffer, pH 7.2. After equilibration and background counts, a non-redox-cycling concentration of lucigenin (5  $\mu$ M) and b-NADPH (12  $\mu$ M) 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 b-NADPH-driven signals and the results were normalized for the dry weight and reported as lucigenin chemiluminescence/mg of dry tissue.

### 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  $\pm$  S.E.M. The comparisons between groups were assessed by two-way analysis of variance using Bonferroni correction (GraphPad Prism 3 Software, San Diego, CA). A probability value  $< 0.05$  was considered significant.

## Results

### Sodium nitrite exerts antihypertensive effect in DOCA-salt hypertension

Hypertension was induced in this study by surgical uninephrectomy (Unx) followed by weekly administration of deoxycorticosterone (or vehicle) along with excess salt in drinking water. Baseline systolic blood pressure (SBP) and body weight were similar in the four experimental groups (Fig. 1A and B;  $P > 0.05$ ). No significant changes of SBP were found the Vehicle and Nitrite groups throughout the study period. However, the DOCA-salt treatment induced a sustained increase in SBP after the first and second weeks of treatment (Fig. 1A). While the SBP increased further with time in the DOCA+vehicle group, daily gavage with sodium nitrite (15 mg/kg) significantly attenuated the increases in the DOCA+nitrite group after five and six weeks of treatment (Fig. 1A;  $P < 0.05$ ).

While body weight doubled in Vehicle and Nitrite groups after 6 weeks of treatment (Fig. 1B;  $P < 0.05$ ), DOCA-treated animals gained less than 25% of their initial weight during the same period (Fig. 1B;  $P < 0.05$ ).

### Sodium nitrite treatment increases plasma nitrite and NO<sub>x</sub> concentrations

Hypertension decreased plasma nitrite and NO<sub>x</sub> concentrations as revealed by lower plasma nitrite and NO<sub>x</sub> levels in the DOCA+vehicle group compared with the Vehicle group (Fig. 2A and B; both  $P < 0.05$ ). However, treatment with sodium nitrite increased plasma nitrite and NO<sub>x</sub> concentrations significantly, both in controls and in DOCA hypertensive rats (Fig. 2A and B; both  $P < 0.05$ ). Interestingly, overall plasma nitrite concentrations remained below 1  $\mu\text{mol/l}$  in animals treated with sodium nitrite.

### Treatment with sodium nitrite decreases the concentrations of systemic markers of oxidative stress and vascular oxidative stress

To assess systemic oxidative stress associated with hypertension and possible antioxidant effects of sodium nitrite, plasma 8-isoprostanes and lipid peroxide levels expressed in terms of

malondialdehyde (MDA) concentrations were measured in plasma samples from rats. Hypertension increased both plasma 8-isoprostane and MDA levels significantly, and treatment with sodium nitrite completely blunted these alterations (both  $P < 0.05$ ; Fig. 3A and B).

To further confirm these alterations in this marker of systemic oxidative stress, reactive oxygen species production *in situ* was evaluated by using the sensitive probe DHE in aortic slices from the animals. In agreement with the systemic marker, we found that DHE oxidation was significantly increased in the hypertensive DOCA+vehicle group compared to the respective control group, and the treatment with sodium nitrite attenuated DOCA-induced oxidative stress (both  $P < 0.05$ ; Fig. 4A and B).

### Treatment with sodium nitrite normalizes vascular NADPH oxidase-dependent reactive oxygen species (ROS) formation in DOCA-salt hypertensive rats

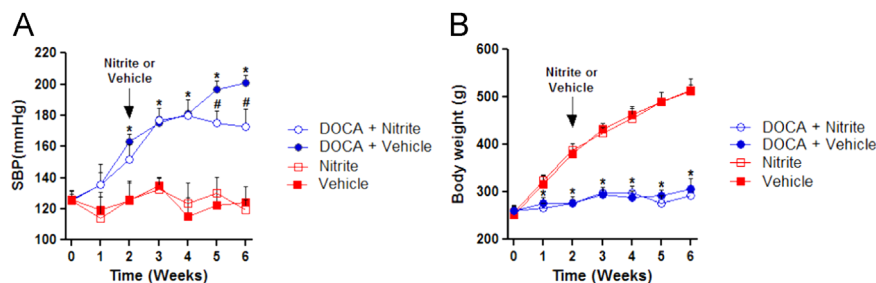
Vascular NADPH oxidase is an important pro-oxidant enzyme, especially in hypertension, and a previous study showed that treatment with sodium nitrite inhibits vascular NADPH oxidase activity in renovascular hypertension [15]. Therefore, NADPH oxidase-dependent ROS formation in aortas from rats were measured using lucigenin chemiluminescence [15]. In agreement with this previous study, we found increased NADPH oxidase activity in hypertensive rats compared with the control group (Fig. 5A;  $P < 0.05$ ), and treatment with sodium nitrite completely blunted hypertension-induced increases in NADPH oxidase activity (Fig. 5A;  $P < 0.05$ ).

### Treatment with sodium nitrite normalizes vascular xanthine oxidoreductase (XOR) aortic activity in DOCA-salt hypertensive rats

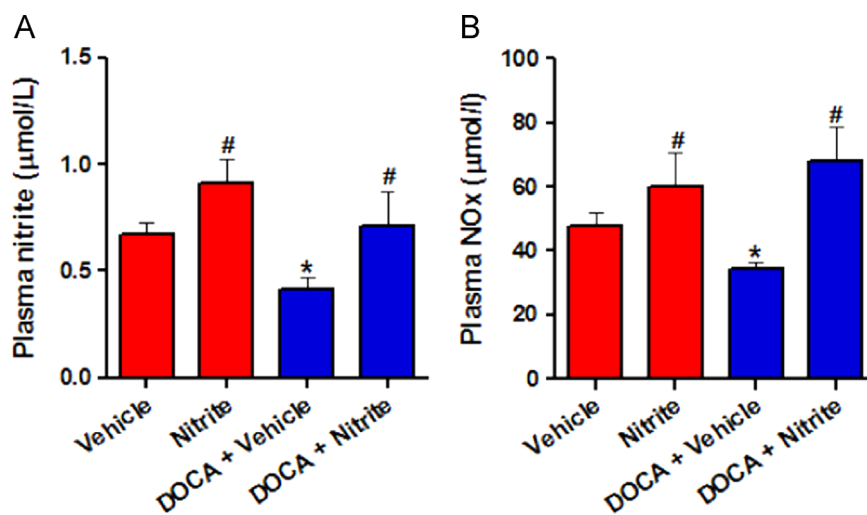
In addition to NADPH oxidase, aortic XOR activity has been shown to contribute to both oxidative stress and to increased vascular relaxing responses to sodium nitrite in hypertensive animals [11]. Therefore we examined whether those modifications would also be found in the DOCA-salt model of hypertension. Consistent with other hypertension models, we found increased aortic XOR activity in DOCA-salt hypertensive rats compared to the control group, and treatment with sodium nitrite normalized XOR activity (Fig. 5B; both  $P < 0.05$ ).

## Discussion

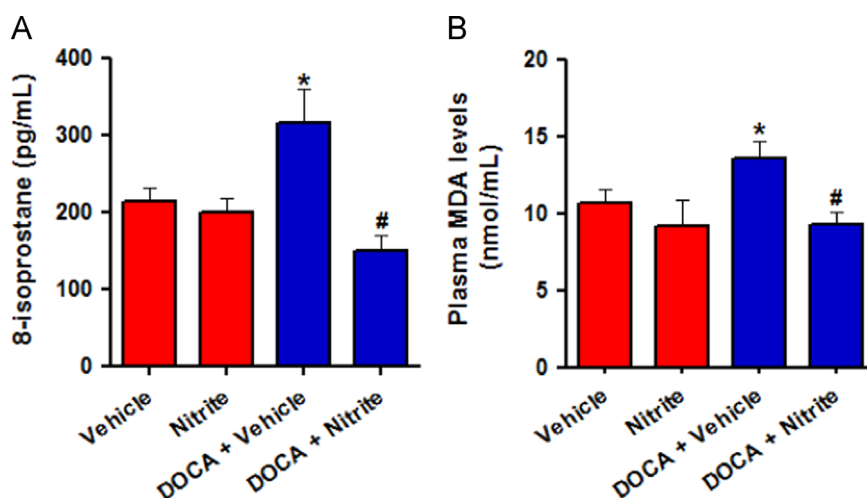
This is the first study to show that oral sodium nitrite administered orally once a day exerts antihypertensive effects in the DOCA-salt hypertension model. Our results clearly show that minor increases in plasma nitrite concentrations were associated with complete blunting of DOCA-salt hypertension-induced systemic and vascular oxidative stress, and of pro-oxidant enzyme



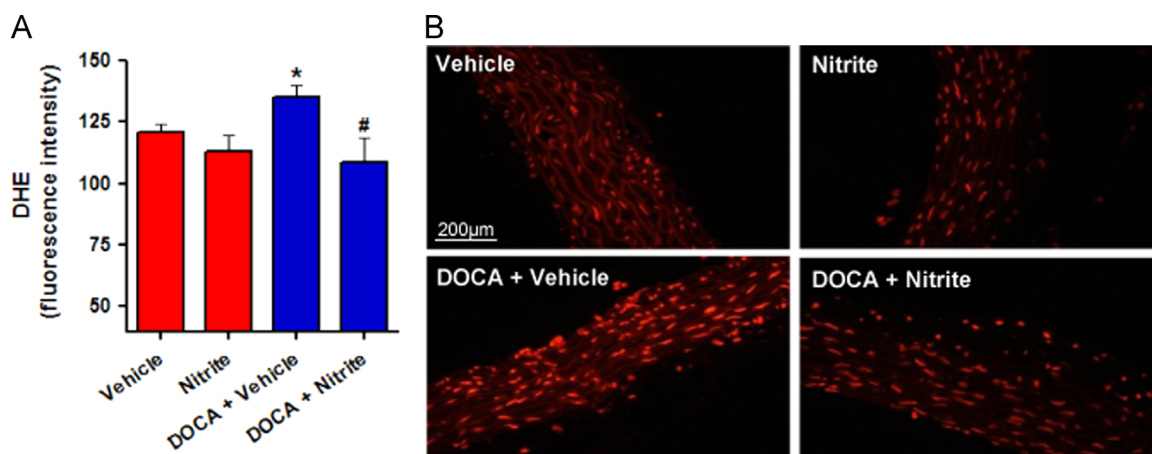
**Fig. 1.** Systolic blood pressure measured by non-invasive tail-cuff method (mmHg; panel A) and body weight changes (g; panel B) throughout the study. (A) Data are shown as mean  $\pm$  S.E.M ( $n=8-10$  per group). \* $P < 0.05$  for DOCA+vehicle versus vehicle group; # $P < 0.05$  for DOCA+nitrite group versus DOCA+vehicle group. (B) \* $P < 0.05$  for DOCA groups versus control groups.



**Fig. 2.** Plasma nitrite and NO<sub>x</sub> levels at end of the treatments. (A) This panel shows plasma nitrite concentration (µmol/l) assessed by reductive chemiluminescence. (B) This panel shows plasma NO<sub>x</sub> concentrations assessed by Griess reaction. Data are shown as mean ± S.E.M. (*n* = 5–7 per group). \**P* < 0.05 Versus vehicle group; #*P* < 0.05 versus respective control group.

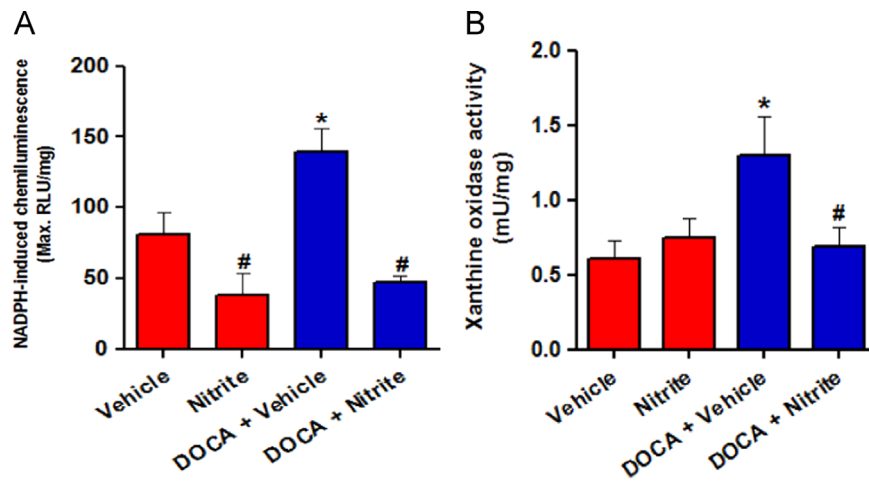


**Fig. 3.** Effects of nitrite treatment on systemic markers of oxidative stress. Panel A shows plasma 8-isoprostanes concentrations (pg/ml) and panel B shows plasma lipoperoxide concentrations expressed in terms of malondialdehyde (MDA) (nmol/ml) in all experimental groups. Data are shown as mean ± S.E.M. (*n* = 4–6 per group). \**P* < 0.05 versus vehicle; #*P* < 0.05 versus DOCA+vehicle group.



**Fig. 4.** Effects of nitrite treatment on *in situ* vascular  $O_2^{\bullet-}$  production assessed by dihydroethidium (DHE) fluorescence. Panel A shows the fluorescence intensity in each experimental group. Data are shown as mean ± S.E.M. (*n* = 5–7 per group). \**P* < 0.05 versus vehicle; #*P* < 0.05 versus DOCA+vehicle group. Panel B shows representative photomicrographs (original magnification × 400) of arteries incubated in the presence of DHE, which produces a red fluorescence when oxidized to hydroxyethidium by  $O_2^{\bullet-}$ .





**Fig. 5.** Sodium nitrite decreases vascular NADPH and xanthine oxidase activities. (A) NADPH oxidase activity in the aortas from rats in all experimental groups was measured by lucigenin chemiluminescence. \* $P < 0.05$  versus vehicle group; # $P < 0.05$  versus vehicle group and DOCA+vehicle group. (B) Xanthine oxidase activity in aortas from rats in all experimental groups. \* $P < 0.05$  versus vehicle group; # $P < 0.05$  versus DOCA+vehicle group. Data are shown as means  $\pm$  S.E.M. ( $n=5$  per group).

activity. Together, our findings are consistent with the idea that treatment with sodium nitrite results in important antioxidant effects [15,16,31] that may contribute to lower blood pressure in hypertension.

Although nitrite has long been viewed as a metabolic product of NO oxidation, this notion has changed dramatically in the last few decades, and nitrite is now accepted as a reservoir of NO that can be released under favorable conditions [7]. With respect to the antihypertensive effects of inorganic nitrites, studies have shown promising effects in different animal models of hypertension [11,15,16,19–24], and it is generally believed that inorganic nitrites reach the circulation and then are reduced to form the potent vasodilator NO by a variety of enzymes or other proteins with nitrite-reductase activity including vascular [15] or erythrocytic [22] XOR, even though a variety of other enzymatic pathways may potentially convert nitrite into NO [1,3]. However, the mechanisms explaining antihypertensive effects of nitrite are still poorly understood, and therefore examining the effects of nitrite in different hypertension models may offer improved mechanistic insights about this issue.

While Classen et al. [37] were first to show antihypertensive effects of nitrite, studies by other groups have confirmed this finding in different models of hypertension [11,15,20,38]. However, no previous study has shown antihypertensive effects of nitrite in the DOCA-salt experimental model, which is a low-renin model, and highly dependent on sodium and increased oxidative stress [25–27]. This is particularly important because increased oxidative stress plays a major role in the DOCA-salt hypertension model, which is less dependent on activation of the renin-angiotensin system than other hypertension models. Interestingly, sodium nitrite exerted major antihypertensive effects in rats with renovascular hypertension (two-kidney, one-clip model), which is a classical renin-dependent hypertension model [26], with increased angiotensin-converting enzyme activity [39,40], even though nitrite therapy apparently does not affect the activity of this important enzyme [21].

The antihypertensive effects shown in the present study agree with those reported after treatment with nitrate (instead of nitrite) in a salt-induced hypertension model [24], and the mechanism explaining the antihypertensive effects may involve antioxidant effects of nitrite on renal microvasculature [16]. Indeed, a recent study showed that nitrite counteracts angiotensin II-induced vasoconstriction of renal afferent arteriole as a result of inhibition of NADPH oxidase activity by nitrite [16]. Interestingly, XOR was involved in this effect because it catalyzed nitrite

reduction to NO [16]. Supporting these previous findings, our results showed that nitrite lowered blood pressure and blunted hypertension-induced increases in vascular NADPH-dependent ROS formation and XOR activity. However, our findings are certainly not explained only by nitrite effects on renal microvasculature. This is because angiotensin II concentrations are not increased in the DOCA-salt model (this is a low-renin model [26]). In fact, we found that treatment with sodium nitrite completely blunted the increases in two relevant markers of systemic oxidative stress (plasma 8-isoprostanes and MDA concentrations), thus indicating that this treatment exerted antioxidant effects not limited to a particular vascular bed, as previously suggested [15,31]. Although we have not studied aortic relaxation, it is possible that nitrite treatment may have improved vascular function in the present study.

Taken together, our results show that sodium nitrite counteracts the oxidative stress associated with DOCA-salt hypertension and may therefore improve NO activity in a variety of vascular beds, thus reducing blood pressure. In fact, the reductions in plasma nitrite and NO<sub>x</sub> concentrations that we found in DOCA-salt hypertensive rats compared with controls indicates that NO bioavailability is decreased in this hypertension model, and restoring systemic and vascular NO bioavailability may improve vascular dysfunction of hypertension and lower blood pressure, as previously shown [35]. Moreover, the important reductions in vascular oxidative stress that we found may prevent the activation of various mechanisms involved in vascular remodeling associated with hypertension, particularly matrix metalloproteinases (MMP) [41]. Indeed, nitrite [42] and other antihypertensive drugs with antioxidant effects [36,43,44] attenuated MMP activation and vascular remodeling of hypertension.

In conclusion, our results show that sodium nitrite exerts antioxidant and antihypertensive effects in the DOCA-salt model of hypertension probably as a result of inhibited NADPH oxidase and XOR activity. Our findings may have relevant implications, not only in terms of lowering blood pressure but also to prevent long term cardiovascular modifications associated with oxidative stress and hypertension [45].

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