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Original article

Oral nitrite circumvents antiseptic mouthwash-induced disruption of enterosalivary circuit of nitrate and promotes nitrosation and blood pressure lowering effect

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ABSTRACT

The nitric oxide (NO•) metabolites nitrite and nitrate exert antihypertensive effects by mechanisms that involve gastric formation of S-nitrosothiols. However, while the use of antiseptic mouthwash (AM) is known to attenuate the responses to nitrate by disrupting its enterosalivary cycle, there is little information about whether AM attenuates the effects of orally administered nitrite. We hypothesized that the antihypertensive effects of orally administered nitrite would not be prevented by AM because, in contrast to oral nitrate, oral nitrite could promote S-nitrosothiols formation in the stomach without intereference by AM. Chronic effects of oral nitrite or nitrate were studied in two-kidney, one-clip (2K1C) hypertensive rats (and normotensive controls) treated with AM (or vehicle) once/day. We found that orally administered nitrite exerts antihypertensive effects that were not affected by AM. This finding contrasts with lack of antihypertensive responses to oral nitrate in 2K1C hypertensive rats treated with AM. Nitrite and nitrate treatments increased plasma nitrites, nitrates, and S-nitrosothiols concentrations. However, while treatment with AM attenuated the increases in plasma nitrite concentrations after both nitrite and nitrate treatments, AM attenuated the increases in S-nitrosothiols in nitrate-treated rats, but not in nitrite-treated rats. Moreover, AM attenuated vascular S-nitrosylation (detected by the SNO-RAC method) after nitrate, but not after nitrite treatment. Significant correlations were found between the hypotensive responses and S-nitrosothiols, and vascular S-nitrosylation levels. These results show for the first time that oral nitrite exerts antihypertensive effects notwithstanding the fact that antiseptic mouthwash disrupts the enterosalivary circulation of nitrate. Our results support a major role for S-nitrosothiols formation resulting in vascular S-nitrosylation as a key mechanism for the antihypertensive effects of both oral nitrite and nitrate.

1. Introduction

Evidence accumulated in the last two decades has changed our view of the nitric oxide (NO•) metabolites nitrite and nitrate [1–3]. Many experimental and clinical studies now clearly show that both anions activate biological mechanisms that protect the cardiovascular system against pathophysiological alterations of disease conditions including hypertension [1,4–8]. In this context, the effects of nitrate were intrinsically associated with its enterosalivary cycle, and a new NO synthase-independent pathway leading to NO• formation has been acknowleged, the nitrate-nitrite-NO pathway [9,10]. The basic knowledge leading to the understanding of this new pathway has been established in studies showing that swallowed nitrite generates NO• under the acidic conditions of the stomach [11,12]. Swallowed nitrite in saliva derives mostly from circulating nitrate, which is actively secreted by salivary glands and reduced to nitrite by oral commensal bacteria [13]. The critical role of oral microbiota in reducing nitrate to nitrite has been widely acknowledged and nitrite can be further reduced to NO by a variety of other enzymes under particular conditons [14,15]. Given the relevance of these findings, dietary nitrate supplementation has now been suggested as an effective therapeutic approach to lower blood pressure in hypertensive patients [6,8].

Recent studies, however, have shown that the use of antibacterial mouthwash may challenge the blood pressure lowering effects of nitrate. In fact, the use of antiseptic mouthwash increased blood pressure in healthy individuals [16] and abolished the blood pressure lowering effects of nitrate in rodents [17,18]. This deleterious effect associated with removal of the oral commensal bacteria has been

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attributed to disruption of nitrate reduction to nitrite by oral bacteria, thus preventing nitrite to achieve the stomach and attenuating the increases in plasma nitrite levels after a nitrate load [19]. While there is no doubt that circulating nitrite can promote arterial and venous dilation under normoxic conditions after nitrite is reduced to $NO^{\bullet}$ by deoxyhemoglobin, deoxymyoglobin or other enzymes with nitrite reductase activity [4,20,21], the mechanisms causing this effect are not fully understood [22,23]. In this respect, the effects of orally administered nitrite have recently been associated with increased gastric formation of S-nitrosothiols, independently of the concentrations of nitrite measured in the plasma [23]. In addition, very similar results were found with orally administered nitrate, thus suggesting that the antihypertensive effects of both nitrite and nitrate [23–25] critically involve the gastric formation of S-nitrosothiols [23], and are independent of increases in plasma nitrite concentrations [24].

In the present study, we hypothesized that treatment with oral nitrite circumvents antiseptic mouthwash-induced disruption of enterosalivary circuit of nitrate and therefore promotes blood pressure lowering effects in hypertensive rats. In addition, given that the use of antiseptic mouthwash severely reduces nitrite concentrations in swallowed saliva achiving the stomach in animals treated with nitrate, we hypothesized that the use of antiseptic mouthwash could impair the increases in plasma S-nitrosothiols after treatment with oral nitrate, but not with oral nitrite. We further examined tissue protein nitrosation to examine the consequences of alterations in S-nitrosothiols levels associated with the use of antiseptic mouthwash.

2. Material and methods

2.1. Animals and hypertension model

The animals used in the present study were handled according to the guiding principles published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the study followed the guidelines of the Ribeirao Preto Medical School, University of Sao Paulo. Male Wistar rats (190–210 g) were obtained from the colony at University of São Paulo and maintained on a 12-h light/dark cycle at room temperature (22–25 °C) with free access to standard rat chow and water.

Two kidney, one clip (2K1C) hypertension was induced as previously described [26,27]. Briefly, the rats were anesthetized with tribromoethanol (250 mg/kg) and had their left renal artery clipped with a silver clip (0.2 mm). Sham-operated control rats underwent the same surgical procedure except for the clip placement. The nonsteroidal anti-inflammatory flunixinemeglumine (2.5 mg/kg, sc, Banamine; Schering Plough, Brazil) was administered after surgery. Systolic blood pressure (SBP) was assessed weekly by tail-cuff plethysmography [25], and the last assessment was carried out approximately 18 h after the last dose of nitrite or nitrate treatments. To minimize the effects of stress induced by this method on blood pressure measurement, the animals were trained for a week before surgery.

To confirm blood pressure measurements with the tail-cuff method, invasive mean arterial pressure (MAP) was evaluated at the end of study period, approximately 6 h after the last dose of nitrite or nitrate treatment. The animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and had their femoral artery cannulated (2 cm segment of a PE-10 tube connected to 14 cm of a PE-50 tubing; Clay Adams, Parsippany, NJ, USA). The catheter was tunneled subcutaneously and exteriorized through the back of the neck. After surgery, the nonsteroidal anti-inflammatory flunixinemeglumine (2.5 mg/kg, s.c., Banamine®, Schering Plough, Brazil) was administered for post-operation analgesia. After 6 h of rest, 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 (Acknowledge 3.2, for Windows). Before collecting data, we allowed at least 15 min of stabilization [28].

2.2. Nitrite or nitrate treatments and the use of antiseptic mouthwash

A first study was designed to examine the effects of antiseptic mouthwash on the antihypertensive effects of sodium nitrite. After two weeks of 2K1C hypertension, both hypertensive and Sham-operated received sodium nitrite 15 mg/kg (or vehicle) daily, by gavage [23,28,29], and had their mouths cleaned with a swab containing a commercial antiseptic mouthwash (Periogard®, chlorhexidine 0.12% or saline) once a day [17]. Nitrite and mouthwash (or respective vehicles) treatments were maintained for additional four weeks in the eight groups of animals (N=10 rats/group). Six hours after the last nitrite (or vehicle) administration, the rats were anesthetized with tribromoethanol (250 mg/kg) and arterial blood samples were collected into heparin containing tubes and immediately centrifuged at 1000*g* for 4 min. Plasma aliquots were mixed with a solution containing N-ethylmaleimide (10 mmol/L) and diethylenetriaminepentaacetic acid (2 mmol/L) to preserve S-nitrosothiols and stored at −70 °C until used to analyze nitrite, nitrate, and S-nitrosothiols concentrations. The aortas were dissected and stored at −70 °C until used to quantify protein nitrosylation.

A second study was designed to examine the effects of antiseptic mouthwash on the antihypertensive effects of sodium nitrate. This study was carried out using the same procedures used in the first study, except that sodium nitrate 140 mg/kg [17,23] was used to replace sodium nitrite (eight groups of animals; N=10 rats/group).

The daily doses of nitrite and nitrate used in the present study were chosen with basis on a series of previous studies by our group showing that these doses significantly reduce blood pressure in 2K1C [5,23,28], L-NAME [30], and DOCA-salt [31] hypertension models. In addition, the doses of both anions used here are the same doses used in a previous study by another group and correspond to pharmacological doses with effects on blood pressure that were prevented by antiseptic mouthwash in the case of nitrate [17]. Both nitrite and nitrate were administered by gavage (1.5 ml/kg of 10 mg/ml and 93 mg/mg, respectively) because we wanted to make sure that all rats received exactly the same dose/body weight.

2.3. Assessment of oral bacterial concentrations

A tongue swab was collected at the end of the study period to assess the concentration of oral bacteria [18]. This number was estimated with basis on the number of colony-forming units (CFU) counted after the bacteria from the swab were smeared on an agar plate and incubated for 18 h before counting the number of colonies.

2.4. Measurement of plasma nitrate, nitrite, and S-nitrosothiols concentrations

Plasma aliquots were analyzed in duplicate for their nitrite and S-nitrosothiols contents using an ozone-based reductive chemiluminescence assay as previously described [28,32]. Briefly, to measure nitrite concentrations in plasma, 50 µl of plasma samples were 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). To measure nitroso compounds (RSNO) concentrations, 500 µl of plasma samples were treated with acid sulfanilamide (5% sulfanilamide in HCl 1 mol/L) for 5 min before injection into the solution of acidified tri-iodide purged with nitrogen in line with the NO analyzer.

The plasma nitrate+nitrite (NOx) concentrations were determined in duplicate by using the Griess reaction as previously described [28], and plasma nitrate concentrations were calculated by subtracting plasma nitrite concentrations from NOx. Briefly, 40 µl of plasma were incubated with the same volume of nitrate reductase buffer (0.1 mol/L

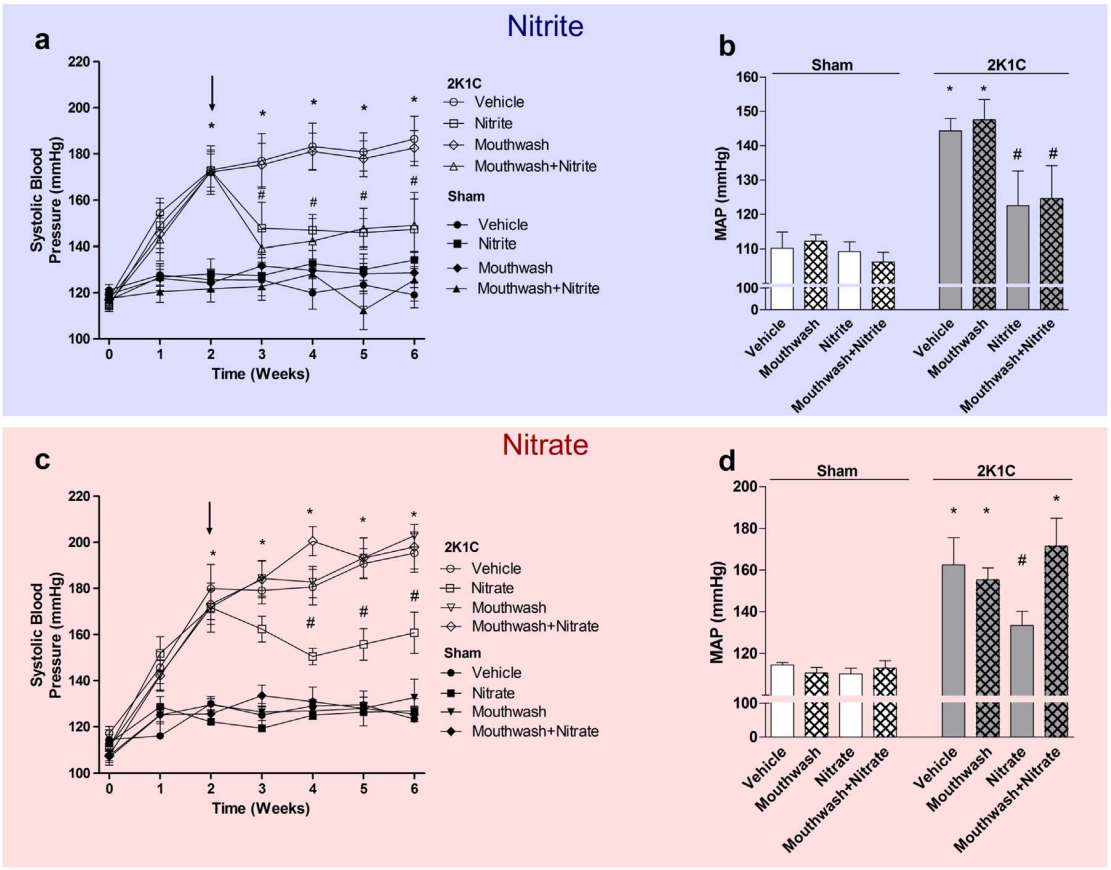

Fig. 1. The antihypertensive responses to sodium nitrite, but not nitrate, resist the mouthwash effects. **a)** Systolic blood pressure in 2K1C hypertensive and Sham-operated rats treated with nitrite (or vehicle) and antiseptic mouthwash (or vehicle). Treatments with nitrite (or vehicle) and mouthwash started after two weeks of hypertension (arrow). **b)** Invasive, mean arterial pressure (MAP) at the end of four weeks of treatments described in a). **c)** Systolic blood pressure in 2K1C hypertensive and Sham-operated rats treated with nitrate (or vehicle) and antiseptic mouthwash (or vehicle). Treatments with nitrate (or vehicle) and mouthwash started after two weeks of hypertension (arrow). **d)** Invasive, mean arterial pressure (MAP) at the end of treatments described in c). Data are shown as mean ± S.E.M. (n=8–10 per group). * $P < 0.05$ *versus* Sham Vehicle group. # $P < 0.05$ *versus* 2K1C Vehicle group.

potassium phosphate, pH 7.5, containing b-nicotinamide adenine dinucleotide phosphate 1 mmol/L and 2U of nitrate reductase/ml) in 96-well plates. The samples were incubated overnight at 37 °C in the dark. Eighty microliters of freshly prepared Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediaminedihydrochloride in 5% phosphoric acid) were added to each well and the plate was incubated for additional 15 min at room temperature. A standard nitrate curve was obtained by incubating sodium nitrate (0.2–200 mol/L) with the same reductase buffer.

2.5. Assessment of protein nitrosylation by resin-assited capture (SNO-RAC) method

Because treatment with oral nitrite or nitrate increased the circulating levels of S-nitrosothiols, which could promote nitrosylation of tissue targets involved in the antihypertensive effects of both anions, we quantified total protein nitrosylation in aortas from rats used in the present study. Total nitrosylated proteins were determined using the SNO-RAC method [33] with modifications. Proteins were extracted from aortic tissue with a buffer (25 mM HEPES, 50 mM NaCl, 0.1 mM EDTA, 1% NP40 and 0.1% SDS pH 7.4) supplemented with protease inhibitor cocktail (Sigmafast™ Sigma) and centrifuged at 12000*g* at 4 °C for 10 min. The supernatant was added to 1.6 ml of blocking buffer (HEN Bufer: 100 mM HEPES, 1 mM EDTA and 0.1 mM neocuproine, pH 8.1 plus 2.5% SDS and 20 mM methylmethanethiosulfonate) for 20 min at 50 °C, mixing every 5 min. Then 6 ml of pre-chilled acetone were added to precipitate the proteins for 20 min at −20 °C. The samples were centrifuged at 2000*g* for 10 min at 4 °C and the pellets were washed four times with acetone 70% and suspended in 0.5 ml of HEN buffer with 1% SDS. Then the samples were incubated overnight at 4 °C with 20 mM ascorbate and 40 µl of thiopropyl-sepharose 6B under rotation. All the steps were carried out in the absence of light. The resin was washed four times with 1 ml of HEN buffer plus 1% SDS and five times with HEN buffer diluted 1:10 with 1% SDS (HEN Buffer/10 SDS) followed by elution with HEN Buffer/10 SDS plus 2% of 2-mercaptoethanol for 1 h at room temperature. To quantify the proportion of nitrosylated proteins, both control input (i) and output (o) samples are run on a 5% SDS/PAGE gel combined with a 10% 5% SDS/PAGE gel. The run was stopped when the samples reached the 10% gel. Then the gels were stained with Coomassie Blue 0.05% and nitrosylated proteins were quantified (Amersham Image 600, GE Healtcare, Little Chalfont, Buckinghamshire, UK) using the ImageJ Program (NIH, USA). Given the low protein concentration in the output samples, we loaded three times higher protein concentration for output samples as compared to input samples. The percentage of protein S-nitrosylation was calculated as 100%×3(i)/(o).

2.6. Drugs and solutions

Thiopropyl-sepharose 6B was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK) and all other drugs and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA). All solutions were prepared immediately before use.

2.7. Statistical analysis

The results are expressed as means ± S.E.M. The comparisons between groups were assessed by one-way or two-way analysis of variance followed by the Tukey test. The Pearson correlation (r, *P*) was calculated for associations between changes in nitrosylated proteins, plasma nitrosothiol concentrations, and systolic blood pressure. A probability value $P < 0.05$ was considered significant.

3. Results

3.1. The antihypertensive responses to sodium nitrite, but not nitrate, resist the mouthwash effects

While a variety of studies has shown antihypertensive effects of both nitrite and nitrate, there is very little information exploring how antiseptic mouthwash affects the antihypertensive responses to nitrite [17]. In agreement with a recent study [23], both nitrite and nitrate decreased SBP by approximately 40 mmHg (both $P < 0.05$; Fig. 1). Importantly, this study shows that the use of mouthwash completely blunted the antihypertensive effects of nitrate ($P < 0.05$; Fig. 1c), without exerting any relevant effect on the antihypertensive responses to nitrite (Fig. 1a). The use of mouthwash alone exerted no significant effects on blood pressure (Fig. 1; all $P > 0.05$).

To confirm SBP results found with the tail-cuff method, we assessed invasive blood pressure. Our MAP results clearly show that both nitrite and nitrate lowered MAP significantly (both $P < 0.05$; Fig. 1b and d). In parallel with non-invasive SBP, we found that the use of mouthwash blunted the antihypertensive effects of nitrate (Fig. 1b; $P > 0.05$), but not nitrite (Fig. 1d; $P < 0.05$).

Fig. 2 shows the effects of mouthwash on the number of CFU found on agar plates. This figure clearly shows that treatment with chlorhexidine mouthwash reduced the number of CFU assessed approximately 24 h after the last mouthwash by 50–70% in all groups as compared with respective control groups, both in nitrite and in nitrate treated rats (Fig. 2a and b; all $P < 0.05$).

3.2. Treatment with mouthwash attenuates the increases in plasma nitrite concentrations after treatment with either sodium nitrate or nitrite

Because the blunting of the antihypertensive effects of nitrate (but not nitrite) by antiseptic mouthwash could be explained by lesser increases in the concentrations of plasma nitrite being converted to $NO^{\bullet}$ in the circulation after nitrate (but not nitrite) treatment, we measured the plasma nitrite concentrations 6 h after the last administration of both treatments. Fig. 3 shows that nitrite treatment increased plasma nitrite concentrations in Sham-operated and in 2K1C hypertensive rats by approximately 1 μmol/L (Fig. 3a; $P < 0.05$), whereas nitrate treatment increased plasma nitrite concentrations in Sham-operated and in 2K1C hypertensive rats by approximately 4–7 μmol/L (Fig. 3c; $P < 0.05$). To our surprise, antiseptic mouthwash attenuated the increases in plasma nitrite concentrations by approximately 25–30% in both nitrite and nitrate-treated groups of Sham-operated and in 2K1C hypertensive rats (Fig. 3a and c; $P < 0.05$). These results show that antiseptic mouthwash causes similar attenuation of the increases in plasma nitrite concentrations after both nitrite and nitrate treatment.

Fig. 3 shows the increases in plasma nitrate concentrations after both treatments. We found that nitrite treatment increased plasma nitrate concentrations in Sham-operated and in 2K1C hypertensive rats by approximately 35–40 μmol/L (Fig. 3b; $P < 0.05$), whereas nitrate treatment increased plasma nitrate concentrations in Sham-operated and in 2K1C hypertensive rats by approximately 60–90 μmol/L (Fig. 3c; $P < 0.05$). Antiseptic mouthwash attenuated the increases in plasma nitrate concentrations by approximately 45% in the nitrite-treated group (Fig. 3b; $P < 0.05$), whereas non significant effects were found in nitrate-treated groups (Fig. 3d; $P < 0.05$).

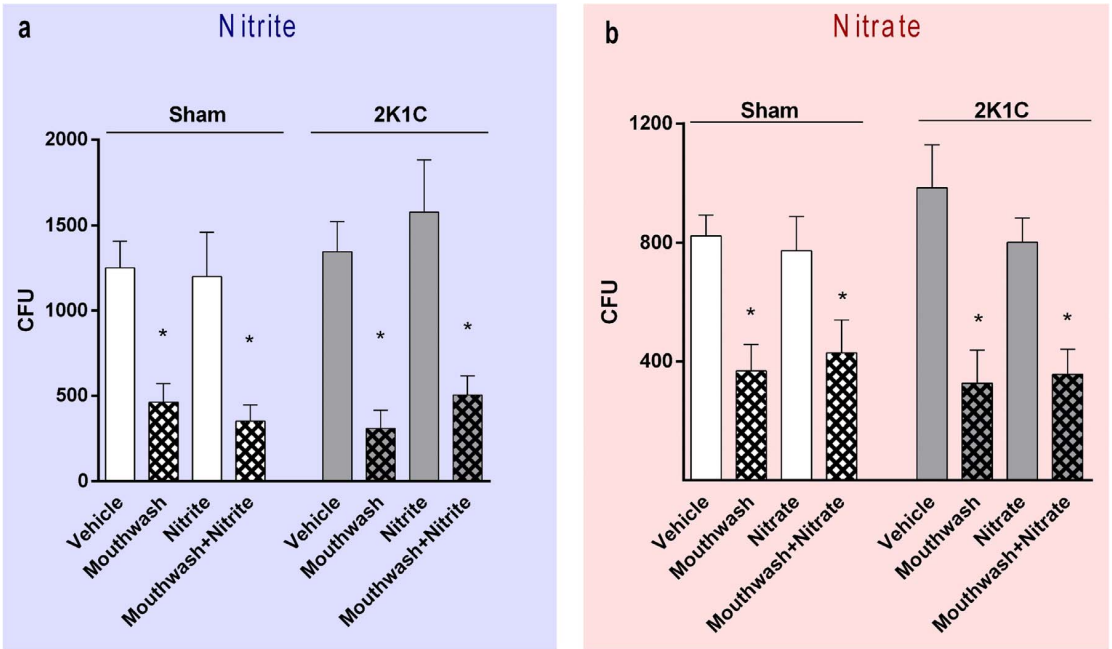

Fig. 2. Treatment with antiseptic mouthwash decreases the number of oral bacteria. The number of colony-forming units (CFU) was counted after the bacteria from the swab were smeared on an agar plate and incubated for 18 h. **a)** CFU in 2K1C hypertensive and Sham-operated rats treated with nitrite (or vehicle) and antiseptic mouthwash (or vehicle). **b)** CFU in 2K1C hypertensive and Sham-operated rats treated with nitrate (or vehicle) and antiseptic mouthwash (or vehicle). Data are shown as mean ± S.E.M. (n=6–9 per group). * $P < 0.05$ *versus* Sham Vehicle group.

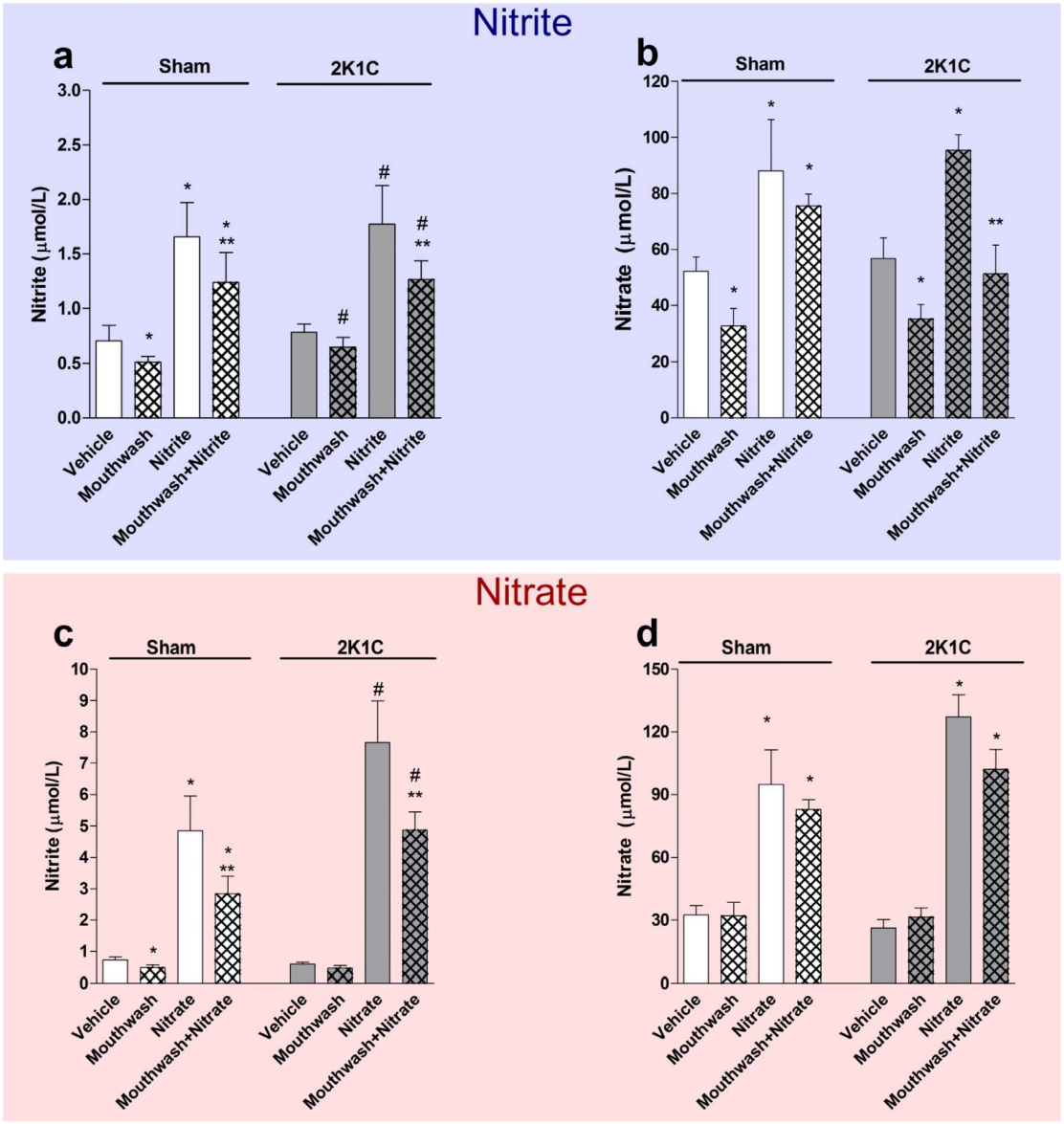

Fig. 3. Antiseptic mouthwash attenuates the increases in plasma nitrite concentrations after treatment with either sodium nitrate or nitrite. a) and b) Plasma nitrite and nitrate concentrations, respectively, in 2K1C hypertensive and Sham-operated rats treated with nitrite (or vehicle) and antiseptic mouthwash (or vehicle). c) and d) Plasma nitrite and nitrate concentrations, respectively, in 2K1C hypertensive and Sham-operated rats treated with nitrate (or vehicle) and antiseptic mouthwash (or vehicle). Data are shown as mean ± S.E.M. (n=6–9 per group). * $P < 0.05$ *versus* Sham Vehicle group. # $P < 0.05$ *versus* 2K1C Vehicle group. ** $P < 0.05$ *versus* respective 2K1C Nitrite or Nitrate group.

3.3. Treatment with mouthwash attenuates the increases in plasma S-nitrosothiols concentrations after treatment with sodium nitrate but not nitrite

Given that the blunting of the antihypertensive effects of nitrate (but not nitrite) by antiseptic mouthwash are not explained by differences in the increases in plasma nitrite levels being converted to $NO^{\bullet}$ after nitrite (*versus* nitrate) treatment, we decided to measure plasma S-nitrosothiols concentrations. This is because oral nitrite administration could circumvent antiseptic mouthwash-induced disruption of enterosalivary circuit of nitrate as a result of nitrite achieving the stomach and promoting the formation of S-nitrosothiols, a mechanism apparently underlying the antihypertensive effects of sodium nitrite [23]. Interestingly, Fig. 4 shows that both nitrite and nitrate treatments increased plasma S-nitrosothiols concentrations by approximately 100% (Fig. 4; both $P < 0.05$). However, antiseptic mouthwash completely blunted the increases in plasma S-nitrosothiols concentrations in rats treated with nitrate (Fig. 4b; $P < 0.05$), but not in rats treated with nitrite (Fig. 4a; $P > 0.05$). These results show that antiseptic mouthwash interfere with the increase in plasma S-nitrosothiols concentrations (Fig. 4) and blunts the antihypertensive effects (Fig. 1) of nitrite, but not nitrate treatment.

3.4. Treatment with mouthwash attenuates the increases in vascular S-nitrosylation levels after treatment with sodium nitrate but not nitrite

The increases in circulating S-nitrosothiols concentrations may mediate antihypertensive effects of orally administered nitrite or nitrate. Therefore, because antiseptic mouthwash blunted the increases in S-nitrosothiols associated with nitrate (but not nitrite) treatment, we examined vascular nitrosylation levels using the SNO-RAC method. Fig. 5 shows that 2k1C hypertension decreases aortic protein nitrosylation by approximately 50% (Fig. 5b and D; both $P < 0.05$), and both

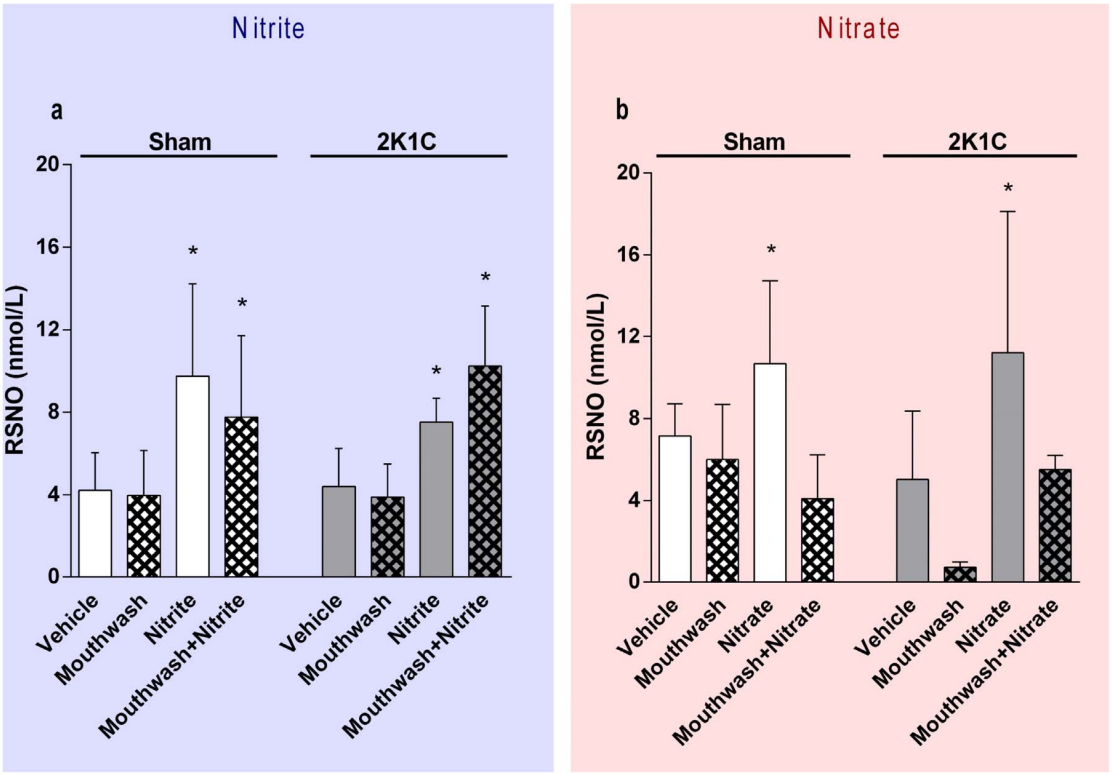

Fig. 4. Antiseptic mouthwash attenuates the increases in plasma S-nitrosothiols concentrations after treatment with sodium nitrate but not nitrite. a) Plasma S-nitrosothiols concentrations in 2K1C hypertensive and Sham-operated rats treated with nitrite (or vehicle) and antiseptic mouthwash (or vehicle). b) Plasma S-nitrosothiols concentrations in 2K1C hypertensive and Sham-operated rats treated with nitrate (or vehicle) and antiseptic mouthwash (or vehicle). Data are shown as mean ± S.E.M. (n=6–9 per group). * $P < 0.05$ *versus* Sham Vehicle group.

nitrite or nitrate treatments do not increase this variable in normotensive Sham-operated rats (Fig. 5b and D; both $P > 0.05$). Interestingly, both nitrite and nitrate treatments almost doubled protein nitrosylation in aortas from 2K1C hypertensive rats (Fig. 5a and b, and Fig. 5c and d, respectively; all $P < 0.05$). However, while antiseptic mouthwash completely prevented vascular S-nitrosylation induced by nitrate treatment (Fig. 5c and d; $P < 0.05$), no significant effects were found in rats treated with nitrite (Fig. 5a and b; $P > 0.05$). These findings show that vascular S-nitrosylation levels are consistent with the S-nitrosothiols concentrations found in plasma samples. Importantly, while both markers increased with either nitrite or nitrate treatments and were associated with antihypertensive effects, antiseptic mouthwash blunted the increases in both markers and the antihypertensive effects exerted by nitrate, but not by nitrite treatment.

3.5. Vascular S-nitrosylation correlates positively with plasma S-nitrosothiol concentrations and negatively with systolic blood pressure

To further support the notion that S-nitrosothiols formation and S-nitrosylation are involved in the antihypertensive effects of both nitrite and nitrate, we examined whether vascular S-nitrosylation correlates with S-nitrosothiols concentrations in plasma and with blood pressure. Interstingly, we found significant positive correlation between S-nitrosothiols concentrations and vascular S-nitrosylation (Fig. 6b; $r=0.97$; $P=0.03$) and negative correlation between vascular S-nitrosylation and blood pressure (Fig. 6a; $r=-0.94$; $P=0.04$) in nitrite-treated animals. Similar correlations were found in nitrate-treated animals, although the association between S-nitrosothiols concentrations and vascular S-nitrosylation was not statistically significant (Fig. 6d; $P=0.18$). These findings support the suggestion that increased concentrations of S-nitrosothiols after nitrite or nitrate treatments promote vascular tissue S-nitrosylation and contribute to the antihypertensive effects of both anions [23]. Moreover, these results indicate that antiseptic mouthwash blunts the antihypertensive effects of nitrate by interfering with S-nitrosylation mechanisms that are not significantly affected when nitrite is used to lower blood pressure.

4. Discussion

This study shows that orally administered nitrite exerts antihypertensive effects in rats treated with antiseptic mouthwash, an effect that contrasts with lack of antihypertensive responses when oral nitrate was used in hypertensive rats treated with the same antiseptic mouthwash. Moreover, this study shows that the antihypertensive responses to either nitrate or nitrate are associated with vascular protein nitrosylation and increased plasma S-nitrosothiols concentrations. Importantly, antiseptic mouthwash prevented blood pressure and nitrosylation responses to nitrate but not to nitrite, thus showing that orally administered nitrite decreases blood pressure independently of the enterosalivary cycle.

Recent studies have shown a variety of protective cardiovascular effects of nitrate and nitrite. Those effects have been explained by nitrite being converted into $NO^{\bullet}$ by several enzymes with nitrite reductase activity including xanthine oxide reductase [30,34–37] and other heme-containing proteins, particularly deoxy-hemoglobin [1,4,38,39]. These mechanisms are certainly very important to generate $NO^{\bullet}$ from nitrite, independent of the route used to administer nitrite or nitrate, which generates nitrite in the oral cavity [10], and therefore part of the antihypertensive effects of both anions are attributable to $NO^{\bullet}$ generation from nitrite. Our results show that plasma nitrite increased after both nitrite and nitrate treatments (Fig. 3), even though lesser increases in nitrite levels were found when the antiseptic mouthwash was used. Interestingly, the increases in

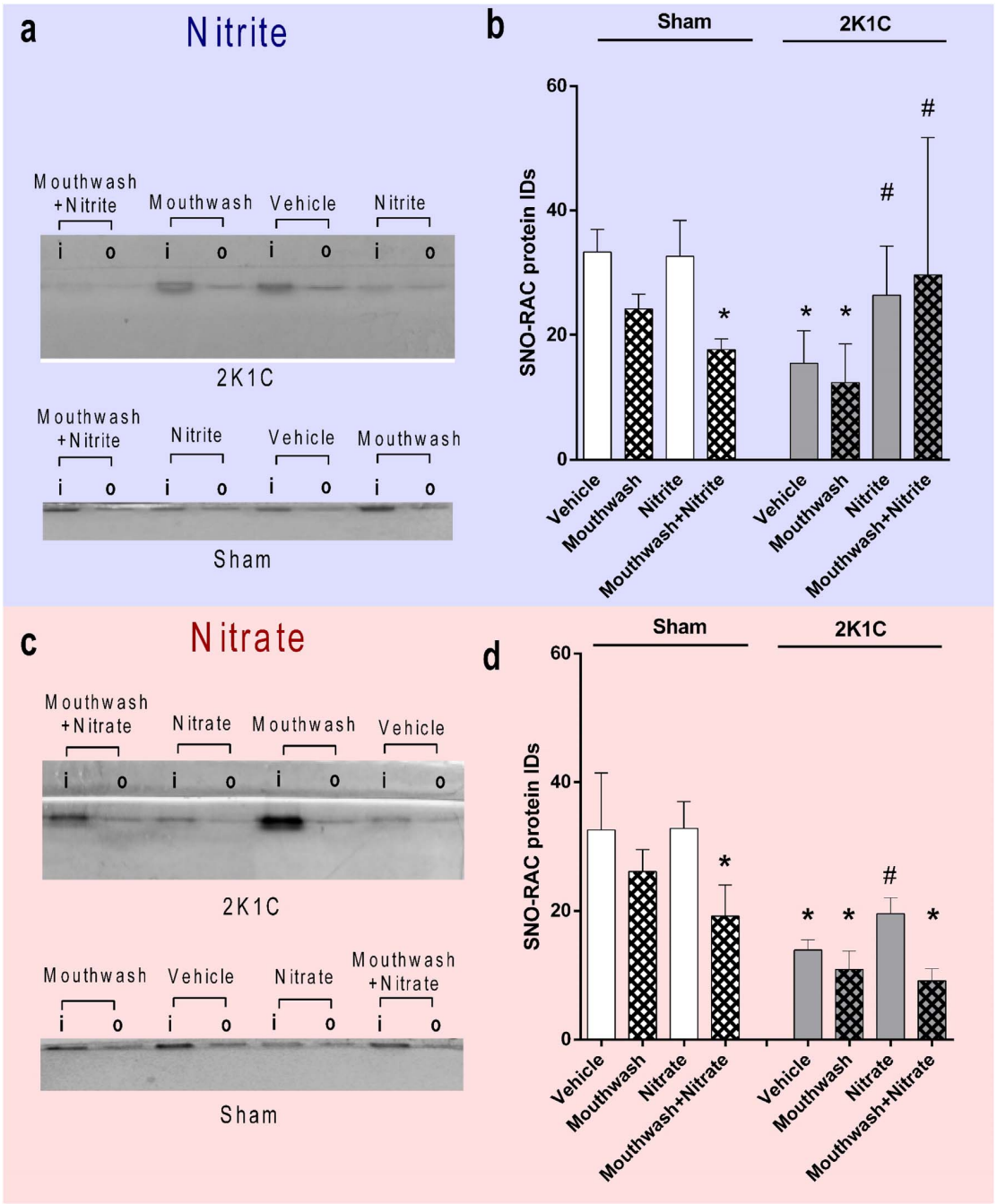

Fig. 5. Antiseptic mouthwash attenuates the increases in vascular S-nitrosylation levels after treatment with sodium nitrate but not nitrite. **a) and c)** Total nitrosylation of aortic proteins determined by the SNO-RAC method using aortas from normotensive and 2K1C hypertensive treated with nitrite (or vehicle) and antiseptic mouthwash (or vehicle), and with nitrate (or vehicle) and antiseptic mouthwash (or vehicle), respectively. Both control input **(i)** and output **(o)** samples are run on a SDS/PAGE gel and stained with Coomassie Blue. **b) and d)** Quantification of S-nitrosylation levels in aortas from Sham-operated (normotensive controls) and 2K1C hypertensive rats treated with nitrite (or vehicle) and antiseptic mouthwash (or vehicle), and with nitrate (or vehicle) and antiseptic mouthwash (or vehicle), respectively. Data are shown as mean ± S.E.M. (n=8 per group). * $P < 0.05$ *versus* Sham Vehicle group. # $P < 0.05$ *versus* 2K1C Vehicle group.

plasma nitrite concentrations following nitrate-treatment has been shown to involve xanthine oxidase activity, which reduces nitrate to nitrite [14].

The lesser increases in plasma nitrite levels after nitrate treatment with the use of antiseptic mouthwash could help to explain how the antiseptic mouthwash prevented the antihypertensive effects of nitrate. It is reasonable to assume that the antiseptic mouthwash attenuated the increases in plasma nitrite concentrations caused by nitrate administration (Fig. 3c), as previously shown [19], and therefore less nitrite is available to generate $NO^{\bullet}$, resulting in attenuation or blunting of the antihypertensive effects of nitrate. However, it is astonishing to note that plasma nitrite increased by approximately 10-fold (from < 0.5 to 5 μmol/L) when nitrate was administered to hypertensive rats treated with antiseptic mouthwash (Fig. 3c), and no antihypertensive effects were found in this group (Fig. 1c and d). These findings clearly indicate that the antihypertensive effects of oral nitrate or nitrite involve mechanisms in addition to the simple increases in circulating nitrite levels and reduction of nitrite to $NO^{\bullet}$ by enzymes or proteins

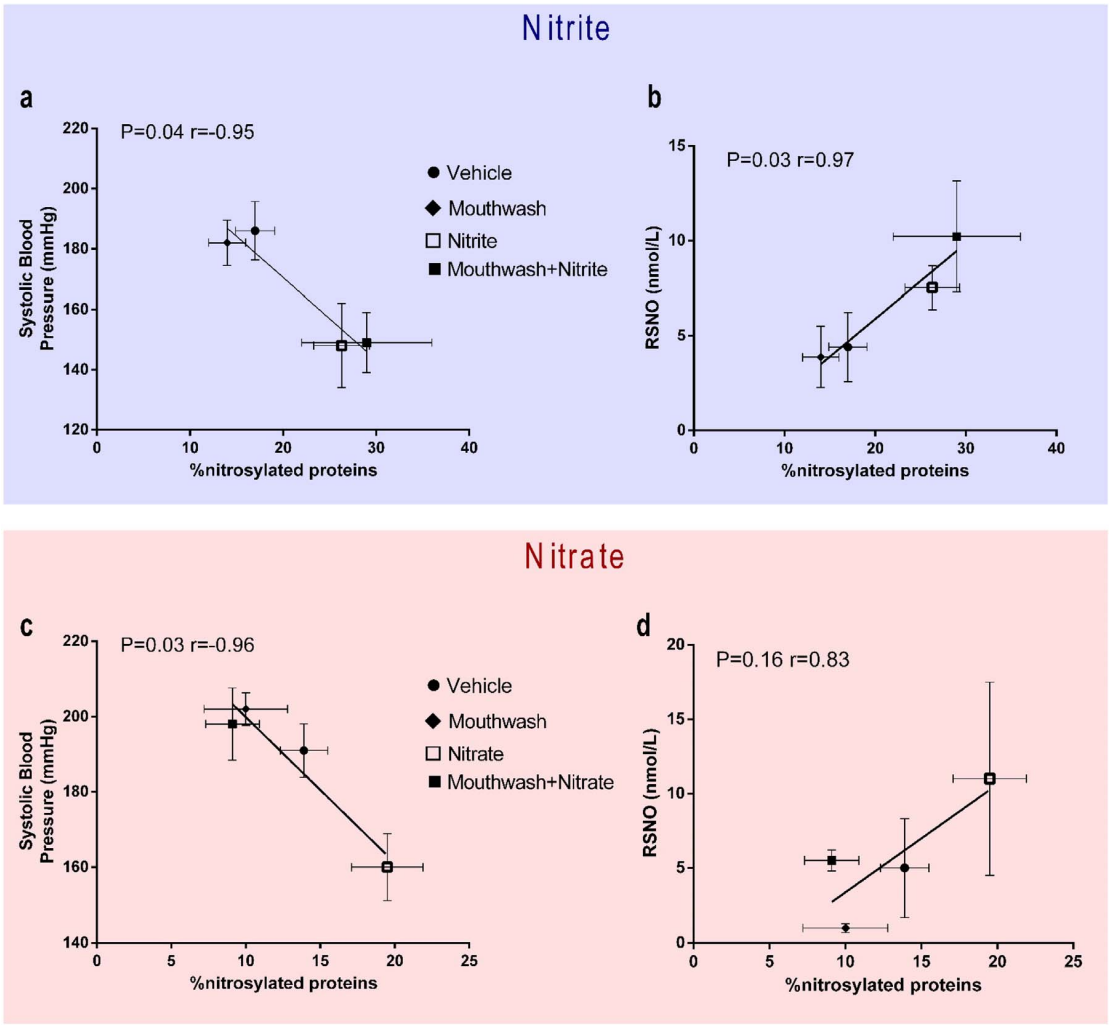

Fig. 6. Vascular S-nitrosylation levels correlate positively with plasma S-nitrosothiols concentrations and negatively with systolic blood pressure. Inverse relationship between systolic blood pressure and vascular protein nitrosylaton (panel a) and direct relationship between plasma S-nitrosothiols concentratons (RSNO) and vascular protein nitrosylaton (panel b) in 2K1C hypertensive rats treated with nitrite (or vehicle) and antiseptic mouthwash (or vehicle). Similar relationships were found in nitrate-treated rats (panels c and d). Data are shown as mean ± S.E.M. (n=6–8 per group). r = Pearson´s correlation coefficient.

with nitrite-reductase activity. Our findings are consistent with the idea that S-nitrosylation is critically involved in the antihypertensive effects of both anions [23], as explained below.

S-nitrosothiols are vasoactive endogenous compounds that mediate vasodilation at relatively low concentrations [40], and increased S-nitrosothiols formation apparently mediate the antihypertensive effects of both orally administered nitrite and nitrate [23]. Nonenzymatic chemical reactions take place in the acidic environment of the stomach and generate $NO^{\bullet}$ from nitrite [11,12,41]. These complex reactions involve nitrite protonation to nitrous acid (HNO_2), which generates a variety of nitrogen oxides including $NO^{\bullet}$ and $NO_2^{\bullet}$ that mediate N- and S-nitrosylation reactions [7,42–44]. Therefore, it is critical that nitrite achieves the gastric cavity to promote these complex reactions and lower blood pressure. In this respect, it has been previously shown that antiseptic mouthwash prevents the reduction of nitrate to nitrite by oral commensal bacteria, disrupts the enterosalivary cycle of nitrate, and abolishes the hypotensive effects of nitrate [17,18]. While our results confirm these previous results with nitrate, we now show that the same effect is not found when nitrite is used instead of nitrate. Indeed, the antihypertensive effects of oral nitrite resisted the deleterious effects of antiseptic mouthwash and treatment with oral nitrite lowered blood pressure independent of the use of antiseptic mouthwash (Fig. 1). Importantly, while both nitrite and nitrate treatments increased circulating S-nitrosothiols concentrations (Fig. 4) and vascular S-nitrosylation levels (Fig. 5) in association with their antihypertensive effects, these biochemical modifications were impaired by antiseptic mouthwash only in nitrate-treated animals, and not in nitrite-treated animals. Taken together, these results provide strong evidence suggesting that the antihypertensive effects of both nitrite and nitrate are probably mediated by mechanisms involving nitrosylation, as previously suggested [23]. The use of antiseptic mouthwash prevents nitrite formation in the oral cavity with no nitrite entering the acidic environment of the stomach after nitrate treatment, thus blunting chemical reactions promoting S-nitrosylation.

It is noteworthy that we found antihypertensive effects of both nitrite and nitrate only when plasma S-nitrosothiols increased. Enhanced gastric formation of S-nitrosothiols, in turn, promotes nitrosylation of many vascular targets by S-transnitrosylation processes [42,45]. In agreement with this idea, we found a direct relationship between S-nitrosothiols concentrations in plasma and vascular S-nitrosylation, and an inverse relationship between vascular S-nitrosylation and blood pressure in nitrite or nitrate-treated animals, independent of the use of antiseptic mouthwash (Fig. 6). These findings suggest that antiseptic mouthwash interferes with blood pressure

responses by inhibiting gastric formation of S-nitrosothiols, which mediated S-nitrosylation of vascular targets. With basis on our results, we it is reasonable to suppose that antiseptic mouthwash prevents nitrate from being reduced to nitrite in the oral cavity, thus preventing nitrite from reaching the gastric cavity, where S-nitrosothiols are generated after nitrite is reduced to NO and other NO-related species. Although we have not determined specific S-nitrosylation targets in the present study, it is possible that the use of antiseptic mouthwash prevented the S-nitrosylation of angiotensin II type 1 receptors (AT1R) after nitrate (but not nitrite) treatment. In support of this suggestion, S-nitrosylation of AT1R has been described as a mechanism decreasing the affinity of angiotensin II for AT1R [46], and activation of the renin-angiotensin system is a major contributor to hypertension in the 2K1C model of hypertension [47].

Interestingly, while S-nitrosothiols concentrations increased to a similar extent in Sham-operated and 2K1C hypertensive rats after treatment with nitrite or nitrate, MAP decreased only in 2K1C hypertensive rats. This could be explained by the fact that antihypertensive effects of drugs are usually proportional to baseline blood pressure. Therefore, the lack of significant effects of nitrate or nitrite treatments on MAP in Sham-operated animals is probably explained by increased sensitivity to nitrite and nitrate therapy in 2K1C hypertensive as compared to normotensive rats, as previously shown [24,28]. On the other hand, given that 2K1C hypertension increases vascular oxidative stress [48], modifications in vascular redox state could change both plasma S-nitrosothiols concentrations and tissue S-nitrosylation [49]. Supporting this suggestion, we found that 2K1C hypertension significantly reduced aortic S-nitrosylation in the present study, thus suggesting that increased oxidative stress commonly found in this animal model of hypertension [50] may impair physiological S-nitrosylation mechanisms that are restored by nitrite or nitrate treatments, as our results show. However, this study has not been designed to address this issue, and therefore further studies are required to clarify these mechanisms.

This study has some limitations to note. The use of antiseptic mouthwash reduced the bacterial counts by approximately 50–70% in the present study, which is similar to previously reported [19]. While it is possible that chlorhexidine antiseptic mouthwash may have increased oral microbiome diversity as a result of chlorhexidine-induced disruption of the abundance of usual bacterial species allowing the proliferation of other species [18], the use of antiseptic mouthwash totally prevented the antihypertensive effects of nitrate. Therefore it remains to be determined whether the use of antiseptic mouthwash disrupts the enterosalivary circulation of nitrate as a result of reduced or altered oral microbiome. It is also surprising that the use of antiseptic mouthwash attenuated the increase in plasma nitrite and nitrate concentrations in nitrite-treated animals. While we have no data to support a more comprehensive explanation for this finding, we could speculated that mouthwash treatment may increase nitrite and nitrate elimination from the body by modifying their metabolism in the gastrointestinal tract or by promoting the formation of other NO-related species. Moreover, we have not studied oxidative stress in the present study, even though oxidative stress plays an important role in the pathophysiology of hypertension and both nitrite and nitrate exert antioxidant effects [5,51]. It is possible that these NO metabolites interfere with the responses to angiotensin II and oxidant stress promoted by this mediator [52]. Treatment with nitrate was shown to attenuate renal NADPH oxidase activity and decreased arteriolar contractions caused by angiotensin II [22]. Another recent study showed that nitrate supplementation improves age-related impairment in endogenous NO generation by inhibiting NADPH oxidase and by modulating the expression of angiotensin II receptors [52]. Finally, the overall number of CFU assessed in the first study (with nitrite) was slightly greater (approximately 40–50%) than the overall number of CFU assessed in the second study (with nitrate)(Fig. 2). Although we do not have a precise explanation for this difference, these two studies were not carried out simultaneously, and therefore environmental conditions may have affected the number of CFU.

In conclusion, this study shows that treatment with oral nitrite circumvents the deleterious effects of antiseptic mouthwash on the antihypertensive effects of oral nitrate, and therefore oral nitrite exerts antihypertensive effects notwithstanding the fact that antiseptic mouthwash disrupts the enterosalivary cicle of nitrate. Our results further support a major role for S-nitrosothiols formation resulting in vascular S-nitrosylation as a key mechanism for the antihypertensive effects of both oral nitrite and nitrate. While antiseptic mouthwash impairs this mechanism by preventing oral nitrate reduction to nitrite, the oral administration of nitrite is not susceptible to this interference and could offer an important alternative to enhance NO signaling [53].

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References

[1] E.E. van Faassen, S. Bahrami, M. Feelisch, N. Hogg, M. Kelm, D.B. Kim-Shapiro, A.V. Kozlov, H. Li, J.O. Lundberg, R. Mason, H. Nohl, T. Rassaf, A. Samouilov, A. Slama-Schwok, S. Shiva, A.F. Vanin, E. Weitzberg, J. Zweier, M.T. Gladwin, Nitrite as regulator of hypoxic signaling in mammalian physiology, Med. Res. Rev. 29 (2009) 683–741.

[2] J.O. Lundberg, M.T. Gladwin, A. Ahluwalia, N. Benjamin, N.S. Bryan, A. Butler, P. Cabrales, A. Fago, M. Feelisch, P.C. Ford, B.A. Freeman, M. Frenneaux, J. Friedman, M. Kelm, C.G. Kevil, D.B. Kim-Shapiro, A.V. Kozlov, J.R. Lancaster Jr, D.J. Lefer, K. McColl, K. McCurry, R.P. Patel, J. Petersson, T. Rassaf, V.P. Reutov, G.B. Richter-Addo, A. Schechter, S. Shiva, K. Tsuchiya, E.E. van Faassen, A.J. Webb, B.S. Zuckerbraun, J.L. Zweier, E. Weitzberg, Nitrate and nitrite in biology, nutrition and therapeutics, Nat. Chem. Biol. 5 (2009) 865–869.

[3] M.T. Gladwin, A.N. Schechter, D.B. Kim-Shapiro, R.P. Patel, N. Hogg, S. Shiva, R.O. Cannon, M. Kelm, D. a. Wink, M.G. Espey, E.H. Oldfield, R.M. Pluta, B. a. Freeman, J.R. Lancaster, M. Feelisch, J.O. Lundberg, The emerging biology of the nitrite anion, Nat. Chem. Biol. 1 (2005), 2005, pp. 308–314.

[4] K. Cosby, K.S. Partovi, J.H. Crawford, R.P. Patel, C.D. Reiter, S. Martyr, B.K. Yang, M.A. Waclawiw, G. Zalos, X. Xu, K.T. Huang, H. Shields, D.B. Kim-Shapiro, A.N. Schechter, R.O. Cannon 3rd, M.T. Gladwin, Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation, Nat. Med. 9 (2003) 1498–1505.

[5] M.F. Montenegro, J.H. Amaral, L.C. Pinheiro, E.K. Sakamoto, G.C. Ferreira, R.I. Reis, D.M.O. Marçal, R.P. Pereira, J.E. Tanus-Santos, Sodium nitrite down-regulates vascular NADPH oxidase and exerts antihypertensive effects in hypertension, Free Radic. Biol. Med. 51 (2011) 144–152.

[6] A.J. Webb, N. Patel, S. Loukogeorgakis, M. Okorie, Z. Aboud, S. Misra, R. Rashid, P. Miall, J. Deanfield, N. Benjamin, R. MacAllister, A.J. Hobbs, A. Ahluwalia, Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite, Hypertension 51 (2008) 784–790.

[7] K. Tsuchiya, Y. Kanematsu, M. Yoshizumi, H. Ohnishi, K. Kirima, Y. Izawa, M. Shikishima, T. Ishida, S. Kondo, S. Kagami, Y. Takiguchi, T. Tamaki, Nitrite is an alternative source of NO in vivo, Am. J. Physiol. Heart Circ. Physiol. 288 (2005) H2163–H2170.

[8] F.J. Larsen, B. Ekblom, K. Sahlin, J.O. Lundberg, E. Weitzberg, Effects of dietary nitrate on blood pressure in healthy volunteers, N. Engl. J. Med. 355 (2006) 2792–2793.

[9] V. Kapil, A.B. Milsom, M. Okorie, S. Maleki-Toyserkani, F. Akram, F. Rehman, S. Arghandawi, V. Pearl, N. Benjamin, S. Loukogeorgakis, R. Macallister, A.J. Hobbs, A.J. Webb, A. Ahluwalia, Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO, Hypertension 56 (2010) 274–281.

[10] J.O. Lundberg, E. Weitzberg, M.T. Gladwin, The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics, Nat. Rev. Drug Discov. 7 (2008) 156–167.

[11] N. Benjamin, F. O'Driscoll, H. Dougall, C. Duncan, L. Smith, M. Golden, H. McKenzie, Stomach NO synthesis, Nature 368 (1994) 502.

[12] J.O. Lundberg, E. Weitzberg, J.M. Lundberg, K. Alving, Intragastric nitric oxide production in humans: measurements in expelled air, Gut 35 (1994) 1543–1546.

[13] J.O. Lundberg, E. Weitzberg, Biology of nitrogen oxides in the gastrointestinal tract, Gut (2012).

[14] E.A. Jansson, L. Huang, R. Malkey, M. Govoni, C. Nihlen, A. Olsson, M. Stensdotter, J. Petersson, L. Holm, E. Weitzberg, J.O. Lundberg, A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis, Nat. Chem. Biol. 4 (2008) 411–417.

[15] L. Huang, S. Borniquel, J.O. Lundberg, Enhanced xanthine oxidoreductase

expression and tissue nitrate reduction in germ free mice, Nitric Oxide 22 (2010) 191–195.
[16] V. Kapil, S.M. a. Haydar, V. Pearl, J.O. Lundberg, E. Weitzberg, A. Ahluwalia, Physiological role for nitrate-reducing oral bacteria in blood pressure control, Free Radic. Biol. Med 55 (2013) 93–100.
[17] J. Petersson, M. Carlström, O. Schreiber, M. Phillipson, G. Christoffersson, A. Jägare, S. Roos, E. a. Jansson, a.E.G. Persson, J.O. Lundberg, L. Holm, Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash, Free Radic. Biol. Med 46 (2009) 1068–1075.
[18] E.R. Hyde, B. Luk, S. Cron, L. Kusic, T. McCue, T. Bauch, H. Kaplan, G. Tribble, J.F. Petrosino, N.S. Bryan, Characterization of the rat oral microbiome and the effects of dietary nitrate, Free Radic. Biol. Med 77 (2014) 249–257.
[19] M. Govoni, E.A. Jansson, E. Weitzberg, J.O. Lundberg, The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash, Nitric Oxide 19 (2008) 333–337.
[20] S. Shiva, Z. Huang, R. Grubina, J. Sun, L.A. Ringwood, P.H. MacArthur, X. Xu, E. Murphy, V.M. Darley-Usmar, M.T. Gladwin, Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration, Circ. Res. 100 (2007) 654–661.
[21] A. Dejam, C.J. Hunter, C. Tremonti, R.M. Pluta, Y.Y. Hon, G. Grimes, K. Partovi, M.M. Pelletier, E.H. Oldfield, R.O. Cannon, A.N. Schechter, M.T. Gladwin, Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation, Circulation 116 (2007) 1821–1831.
[22] X. Gao, T. Yang, M. Liu, M. Peleli, C. Zollbrecht, E. Weitzberg, J.O. Lundberg, A.E. Persson, M. Carlstrom, NADPH oxidase in the renal microvasculature is a primary target for blood pressure-lowering effects by inorganic nitrate and nitrite, Hypertension 65 (2015) 161–170.
[23] L.C. Pinheiro, J.H. Amaral, G.C. Ferreira, R.L. Portella, C.S. Ceron, M.F. Montenegro, J.C. Toledo Jr, J.E. Tanus-Santos, Gastric S-nitrosothiol formation drives the antihypertensive effects of oral sodium nitrite and nitrate in a rat model of renovascular hypertension, Free Radic. Biol. Med. 87 (2015) 252–262.
[24] M.F. Montenegro, L.C. Pinheiro, J.H. Amaral, D.M.O. Marçal, A.C.T. Palei, A.J. Costa-Filho, J.E. Tanus-Santos, Antihypertensive and antioxidant effects of a single daily dose of sodium nitrite in a model of renovascular hypertension, Naunyn Schmiede. Arch. Pharm. 385 (2012) 509–517.
[25] L.C. Pinheiro, J.H. Amaral, G.C. Ferreira, M.F. Montenegro, G.H. Oliveira-Paula, J.E. Tanus-Santos, The antihypertensive effects of sodium nitrite are not associated with circulating angiotensin converting enzyme inhibition, Nitric Oxide 40C (2014) 52–59.
[26] C.S. Ceron, E. Rizzi, D.A. Guimaraes, A. Martins-Oliveira, R.F. Gerlach, J.E. Tanus-Santos, Nebivolol attenuates prooxidant and profibrotic mechanisms involving TGF-beta and MMPs, and decreases vascular remodeling in renovascular hypertension, Free Radic. Biol. Med. 65C (2013) 47–56.
[27] A. Martins-Oliveira, M.M. Castro, D.M. Oliveira, E. Rizzi, C.S. Ceron, D. Guimaraes, R.I. Reis, C.M. Costa-Neto, D.E. Casarini, A.A. Ribeiro, R.F. Gerlach, J.E. Tanus-Santos, Contrasting effects of aliskiren versus losartan on hypertensive vascular remodeling, Int. J. Cardiol. 167 (2013) 1199–1205.
[28] L.C. Pinheiro, M.F. Montenegro, J.H. Amaral, G.C. Ferreira, A.M. Oliveira, J.E. Tanus-Santos, Increase in gastric pH reduces hypotensive effect of oral sodium nitrite in rats, Free Radic. Biol. Med. 53 (2012) 701–709.
[29] J.H. Amaral, M.F. Montenegro, L.C. Pinheiro, G.C. Ferreira, R.P. Barroso, A.J. Costa-Filho, J.E. Tanus-Santos, TEMPOL enhances the antihypertensive effects of sodium nitrite by mechanisms facilitating nitrite-derived gastric nitric oxide formation, Free Radic. Biol. Med. 65C (2013) 446–455.
[30] M.F. Montenegro, L.C. Pinheiro, J.H. Amaral, G.C. Ferreira, R.L. Portella, J.E. Tanus-Santos, Vascular xanthine oxidoreductase contributes to the antihypertensive effects of sodium nitrite in L-NAME hypertension, Naunyn Schmiede. Arch. Pharm. 387 (2014) 591–598.
[31] J.H. Amaral, G.C. Ferreira, L.C. Pinheiro, M.F. Montenegro, J.E. Tanus-Santos, Consistent antioxidant and antihypertensive effects of oral sodium nitrite in DOCA-salt hypertension, Redox Biol. 5 (2015) 340–346.
[32] M. Feelisch, T. Rassaf, S. Mnaimneh, N. Singh, N.S. Bryan, D. Jourd'heuil, M. Kelm, Concomitant S-, N-, and heme-nitros (yl) ation in biological tissues and fluids: implications for the fate of NO in vivo, FASEB J. (2002) 1775–1785.
[33] C. Figueiredo-Freitas, R.A. Dulce, M.W. Foster, J. Liang, A.M. Yamashita, F.L. Lima-Rosa, J.W. Thompson, M.A. Moseley, J.M. Hare, L. Nogueira, M.M. Sorenson, J.R. Pinto, S-nitrosylation of sarcomeric proteins depresses myofilament Ca2+)sensitivity in intact cardiomyocytes, Antioxid. Redox Signal. 23 (2015) 1017–1034.
[34] S.M. Ghosh, V. Kapil, I. Fuentes-Calvo, K.J. Bubb, V. Pearl, A.B. Milsom, R. Khambata, S. Maleki-Toyserkani, M. Yousuf, N. Benjamin, A.J. Webb, M.J. Caulfield, A.J. Hobbs, A. Ahluwalia, Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential, Hypertension 61 (2013) 1091–1102.
[35] H. Li, A. Samouilov, X. Liu, J.L. Zweier, Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrate reduction: evaluation of its role in nitrite and nitric oxide generation in anoxic tissues, Biochemistry 42 (2003) 1150–1159.
[36] H. Li, A. Samouilov, X. Liu, J.L. Zweier, Characterization of the effects of oxygen on xanthine oxidase-mediated nitric oxide formation, J. Biol. Chem. 279 (2004) 16939–16946.
[37] J.L. Zweier, H. Li, A. Samouilov, X. Liu, Mechanisms of nitrite reduction to nitric oxide in the heart and vessel wall, Nitric Oxide 22 (2010) 83–90.
[38] A. Dejam, C.J. Hunter, M.M. Pelletier, L.L. Hsu, R.F. Machado, S. Shiva, G.G. Power, M. Kelm, M.T. Gladwin, A.N. Schechter, Erythrocytes are the major intravascular storage sites of nitrite in human blood, Blood 106 (2005) 734–739.
[39] W.F. Alzawahra, M.A. Talukder, X. Liu, A. Samouilov, J.L. Zweier, Heme proteins mediate the conversion of nitrite to nitric oxide in the vascular wall, Am. J. Physiol. Heart Circ. Physiol. 295 (2008) H499–H508.
[40] T. Liu, H.J. Schroeder, S.M. Wilson, M.H. Terry, M. Romero, L.D. Longo, G.G. Power, A.B. Blood, Local and systemic vasodilatory effects of low molecular weight S-nitrosothiols, Free Radic. Biol. Med. 91 (2016) 215–223.
[41] G.M. McKnight, L.M. Smith, R.S. Drummond, C.W. Duncan, M. Golden, N. Benjamin, Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans, Gut 40 (1997) 211–214.
[42] K. a. Broniowska, N. Hogg, The chemical biology of S-nitrosothiols, Antioxid. Redox Signal. 17 (2012) 969–980.
[43] M.W. Foster, T.J. McMahon, J.S. Stamler, S-nitrosylation in health and disease, Trends Mol. Med. 9 (2003) 160–168.
[44] G. Richardson, S.L. Hicks, S. O'Byrne, M.T. Frost, K. Moore, N. Benjamin, G.M. McKnight, The ingestion of inorganic nitrate increases gastric S-nitrosothiol levels and inhibits platelet function in humans, Nitric Oxide 7 (2002) 24–29.
[45] Y. Zhang, N. Hogg, The mechanism of transmembrane S-nitrosothiol transport, Proc. Natl. Acad. Sci. USA 101 (2004) 7891–7896.
[46] P.C. Leclerc, P.M. Lanctot, M. Auger-Messier, E. Escher, R. Leduc, G. Guillemette, S-nitrosylation of cysteine 289 of the AT1 receptor decreases its binding affinity for angiotensin II, Br. J. Pharm. 148 (2006) 306–313.
[47] C.S. Ceron, E. Rizzi, D.A. Guimaraes, A. Martins-Oliveira, S.B. Cau, J. Ramos, R.F. Gerlach, J.E. Tanus-Santos, Time course involvement of matrix metalloproteinases in the vascular alterations of renovascular hypertension, Matrix Biol. 31 (2012) 261–270.
[48] L.C. Pinheiro, G.H. Oliveira-Paula, R.L. Portella, D.A. Guimaraes, C.D. de Angelis, J.E. Tanus-Santos, Omeprazole impairs vascular redox biology and causes xanthine oxidoreductase-mediated endothelial dysfunction, Redox Biol. 9 (2016) 134–143.
[49] B.A. Maron, S.S. Tang, J. Loscalzo, S-nitrosothiols and the S-nitrosoproteome of the cardiovascular system, Antioxid. Redox Signal. 18 (2013) 270–287.
[50] M.M. Castro, E. Rizzi, G.J. Rodrigues, C.S. Ceron, L.M. Bendhack, R.F. Gerlach, J.E. Tanus-Santos, Antioxidant treatment reduces matrix metalloproteinase-2-induced vascular changes in renovascular hypertension, Free Radic. Biol. Med. 46 (2009) 1298–1307.
[51] M. Carlstrom, A.E. Persson, E. Larsson, M. Hezel, P.G. Scheffer, T. Teerlink, E. Weitzberg, J.O. Lundberg, Dietary nitrate attenuates oxidative stress, prevents cardiac and renal injuries, and reduces blood pressure in salt-induced hypertension, Cardiovasc. Res. 89 (2011) 574–585.
[52] M. Hezel, M. Peleli, M. Liu, C. Zollbrecht, B.L. Jensen, A. Checa, A. Giulietti, C.E. Wheelock, J.O. Lundberg, E. Weitzberg, M. Carlstrom, Dietary nitrate improves age-related hypertension and metabolic abnormalities in rats via modulation of angiotensin II receptor signaling and inhibition of superoxide generation, Free Radic. Biol. Med. 99 (2016) 87–98.
[53] J.O. Lundberg, M.T. Gladwin, E. Weitzberg, Strategies to increase nitric oxide signalling in cardiovascular disease, Nat. Rev. Drug Discov. 14 (2015) 623–641.