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Research Paper

Nitrate decreases xanthine oxidoreductase-mediated nitrite reductase activity and attenuates vascular and blood pressure responses to nitrite

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ABSTRACT

Nitrite and nitrate restore deficient endogenous nitric oxide (NO) production as they are converted back to NO, and therefore complement the classic enzymatic NO synthesis. Circulating nitrate and nitrite must cross membrane barriers to produce their effects and increased nitrate concentrations may attenuate the nitrite influx into cells, decreasing NO generation from nitrite. Moreover, xanthine oxidoreductase (XOR) mediates NO formation from nitrite and nitrate. However, no study has examined whether nitrate attenuates XOR-mediated NO generation from nitrite. We hypothesized that nitrate attenuates the vascular and blood pressure responses to nitrite either by interfering with nitrite influx into vascular tissue, or by competing with nitrite for XOR, thus inhibiting XOR-mediated NO generation. We used two independent vascular function assays in rats (aortic ring preparations and isolated mesenteric arterial bed perfusion) to examine the effects of sodium nitrate on the concentration-dependent responses to sodium nitrite. Both assays showed that nitrate attenuated the vascular responses to nitrite. Conversely, the aortic responses to the NO donor DETANONOate were not affected by sodium nitrate. Further confirming these results, we found that nitrate attenuated the acute blood pressure lowering effects of increasing doses of nitrite infused intravenously in freely moving rats. The possibility that nitrate could compete with nitrite and decrease nitrite influx into cells was tested by measuring the accumulation of nitrogen-15-labeled nitrite (^{15}N-nitrite) by aortic rings using ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS). Nitrate exerted no effect on aortic accumulation of ^{15}N-nitrite. Next, we used chemiluminescence-based NO detection to examine whether nitrate attenuates XOR-mediated nitrite reductase activity. Nitrate significantly shifted the Michaelis Menten saturation curve to the right, with a 3-fold increase in the Michaelis constant. Together, our results show that nitrate inhibits XOR-mediated NO production from nitrite, and this mechanism may explain how nitrate attenuates the vascular and blood pressure responses to nitrite.

1. Introduction

Nitric oxide (NO) has long been valued as a major player in a variety of relevant functions, particularly in the regulation of the cardiovascular system [1]. This is so important that NO deficiency is clearly implicated in many cardiovascular diseases [1], and pharmacological approaches that increase NO activity protect the cardiovascular system against pathophysiological mechanisms promoting disease [2,3]. In this regard, evidence has accumulated in the last decade to support the idea of utilizing the anions nitrite and nitrate to improve or restore deficient endogenous NO production [4,5]. While those anions used to be considered as inactive products of NO metabolism, it is now widely acknowledged that both can be converted back to NO under certain conditions, and therefore complement the classic enzymatic synthesis of NO by NO synthases [4,6]. In fact, many experimental and clinical studies have shown beneficial cardiovascular effects of both anions, even though the precise mechanisms explaining their effects are not entirely known [4,7,8].

Circulating nitrate and nitrite anions must cross membrane barriers to produce their effects, and this requires anion channels. In fact, it has been suggested that passive transport mechanisms probably regulate the increases in nitrite levels, particularly after the administration of acute doses [9]. However, no previous study has tested the possibility that increased nitrate concentrations usually found in plasma and in

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tissues could attenuate the nitrite influx into cells and thus NO generation from nitrite.

Although nitrite itself may directly activate signaling pathways resulting in biological responses [9,10], the possibility that nitrite is reduced to NO by enzymes with nitrite-reductase activity is accepted as an important mechanism to explain its effects [5]. As an important example, xanthine oxidoreductase (XOR) has been shown to mediate the antihypertensive effect of nitrite [11,12], which is attributable, at least in part, to XOR-mediated NO generation [13,14]. Interestingly, in addition to mediating the responses to nitrite, XOR can also mediate the responses to nitrate. This is because XOR can reduce nitrate to nitrite and then to NO [14–16], and this nitrate-reductase activity may also contribute to increased nitrite concentrations after nitrate administration [17]. However, although both nitrite and nitrate are now widely accepted as substrates for XOR leading to NO formation, no previous study has examined whether these anions compete for XOR activity, so that increased tissue or circulating nitrate concentrations could attenuate NO generation from nitrite.

In this study, we hypothesized that increasing nitrate concentrations could attenuate the vascular and blood pressure responses to nitrite by either interfering with nitrite influx into vascular tissue. Alternatively, nitrate could compete with nitrite for XOR and therefore inhibit XOR-mediated nitrite reductase activity and NO generation from nitrite.

2. Materials and methods

2.1. Animals and reagents

The procedures described in this manuscript were approved by the Ribeirao Preto Medical School, University of São Paulo, and followed the guiding principles published by the National Institutes of Health Guide for Care and Use of Laboratory Animals. Male wistar rats (250–400 g) were requested from the colony at University of São Paulo (Ribeirão Preto Campus, Brazil) and maintained on a 12-h light/dark cycle at a room temperature (22–25 °C) with free access to standard rat chow and water.

All reagents used in the present study were purchased from Sigma Chemical Co. (St Louis, MO, USA), except for diethylenetriamine NONOate (DETANONOate; Cayman Chemical; Ann Arbor, MI, USA), and nitrogen-15-labeled nitrite (^{15}N-nitrite; Cambridge Isotopes Laboratories, Inc.; Andover, MA, USA).

2.2. Effects of nitrate on aortic reactivity in response to cumulative concentrations of nitrite

To examine whether increased nitrate concentrations could attenuate the vascular responses to nitrite, we studied vascular relaxation in aortic rings. The thoracic aortas were isolated, cleaned of adherent connective tissue and cut into 4 mm long segment rings. The rings were placed in bath chambers containing modified Krebs salt solution containing (mmol/l): NaCl 118, $CaCl_2{\cdot}2H_2O$ 2.5, $MgSO_4{\cdot}7H_2O$ 1.64, KH_2PO_4 1.2, KCl 4.7, $NaHCO_3$ 24.9 and glucose 11.1, maintained at 37 °C, pH 7.4, bubbled with 95% O_2 and 5% CO_2. Pretension of 1.5 g was used. Isometric measurement of force displacement was assessed by a transducer (Letica Scientific Instruments, Barcelona, Spain) and recorded using the LabChart, PowerLab ADInstruments program. After equilibration, integrity of endothelial function was evaluated by the degree of relaxation induced by acetylcholine (10^{-6} mol/l) following contraction induced by phenylephrine (10^{-7} mol/l). Aortic rings with intact functional endothelium were precontracted with phenylephrine (10^{-7} mol/l) and relaxing response curves to cumulative concentrations (from 3×10^{-8} to 10^{-2} mol/l) of sodium nitrite were constructed in the presence of nitrate 200 or 300 μmol/l or vehicle. The results were expressed as pD_2 (the negative logarithm of the concentration producing half maximum effect) and maximum effect in response to sodium nitrite [18].

2.3. Effects of nitrate on mesenteric arterial bed responses to increasing concentrations of nitrite

Given that most of the systemic resistance to blood flow affecting blood pressure is arteriolar resistance from small arterioles, we further examined whether increased nitrate concentrations could attenuate the vascular responses to nitrite using a mesenteric arterial bed perfusion preparation [19]. After an abdominal incision, the ileocolic, right colic and pancreaticoduodenal arteries were tied off and the superior mesenteric artery was cannulated, and then the gut was removed. The mesenteric bed was cleaned of the blood by perfusing with Krebs solution (as described above) containing heparin 500 IU and the end of mesenteric artery was tied off. The intestinal loop was removed, and the mesenteric arterial bed was coupled by the cannula to a perfusion pump. The preparation was perfused continuously with a 4 ml/min flow (Krebs solution containing ethylenediaminetetraacetic acid 0.03 mmol/l, 37 °C, bubbled with 95% O_2 and 5% CO_2). The perfusion pressure in mesenteric bed was recorded with a data acquisition system (MP150CE; Biopac Systems Inc., CA, USA). The mesenteric arterial bed was contracted by continuous infusion of phenylephrine (3–8 μmol/l) to increase baseline perfusion pressure up to 50–80% of the maximal contraction. After stabilization, sodium nitrate 300 μM or vehicle (H_2O) was added to the perfusion solution. Six minutes later, a dose-response curve to sodium nitrite was constructed by adding increasing concentrations of sodium nitrite (from 0.1 μmol/l to 1 mmol/l) to the perfusion solution to assess the relaxing to sodium nitrite.

2.4. Effects of nitrate on in vivo blood pressure responses to cumulative doses of nitrite in freely moving rats

To further validate sodium nitrate-induced functional changes found in vascular responses to sodium nitrite, we examined whether sodium nitrate could attenuate the in vivo blood pressure responses to increasing doses of sodium nitrite. Rats had their femoral artery and vein cannulated (for blood pressure measurement and drug administration, respectively) with PE-10 tubes connected to PE-50 tubes and tunneled subcutaneously so that they were exteriorized behind the neck. At the end of surgery, the rats received nonsteroidal anti-inflammatory (flunixineMeglumine, 2.5 mg/Kg, sc) for postoperative analgesia. Six hours after surgery, the arterial cannula was connected to a pressure transducer and the mean arterial pressure (MAP) in freely moving rat was recorded by a data acquisition system (MP150CE; Biopac Systems Inc., CA, USA) connected to a computer (Acknowledge 3.8 for Windows).

After baseline MAP assessment, the rats received Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME) 100 mg/Kg orally to increase the MAP. To avoid variability in the hypertensive responses to L-NAME, we included in the present study only those rats with MAP ≥135 mmHg after L-NAME administration. Forty min after L-NAME administration, the animals received nitrate (40 μmol/kg) or saline (controls) intravenously. Ten minutes later, increasing doses of sodium nitrite were infused intravenously (1, 3, 10, 30 and 100 μmol/Kg) every ten min (between dose interval). Finally, 15 after the last dose of sodium nitrite, the animals were anesthetized with tribromoethanol (250 mg/kg) and arterial blood samples were collected into heparin-containing tubes, centrifuged at 3000*g* for 4 min, and the plasma was used in biochemical analysis [20].

2.5. Measurement of plasma nitrite and nitrate concentrations

Plasma samples were analyzed for their nitrite contents by using ozone-based reductive chemiluminescence assay as previously described [21]. Briefly, plasma samples (50 μl) were injected into a

solution of acidified tri-iodide (2 g potassium iodide and 1.3 g iodine dissolved in 40 ml water with 140 ml acetic acid), purged with nitrogen in-line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO analyzer, Boulder, CO, USA). Plasma nitrate + nitrite (NO_X) concentrations were measured by using the Griess reaction, as previously described [22]. Briefly, 40 µl of plasma samples and a standard nitrate curve were incubated with the same volume of nitrate reductase buffer (0.1 mol/l potassium phosphate, pH 7.5, containing 1 mmol/l β-nicotinamide adenine dinucleotide phosphate and 2 U/ml of nitrate reductase) in 96-well plates. After overnight incubation at 37 °C in the dark, 80 µL of Griess reagent (1% sulfanilamide, 0.1% naphthyl-ethylenediamine dihydrochloride in 5% phosphoric acid) were added to each well and the plate was incubated for 15 min at room temperature. NOX concentrations were determined by measuring absorbance at 540 nm using a microplate reader. Plasma nitrate concentrations were calculated by subtracting the concentrations of plasma nitrite from the NO_X concentrations.

2.6. Determination of the effects of sodium nitrate on vascular influx and accumulation of nitrite labeled with stable isotope (^{15}N-nitrite)

Because the attenuation of the responses to nitrite by nitrate could be explained by a competitive mechanism between both anions in such a way that nitrate could attenuate nitrite influx into cells, thus impairing NO generation from nitrite, we designed experiments using nitrogen-15-labeled nitrite (^{15}N-nitrite). Aortic rings from wistar rats were isolated and sectioned into four 5 mm segment rings, which were incubated individually in bath chambers containing Krebs solution (described above) at 37 °C, pH 7.4, bubbled with 95% O_2 and 5% CO_2. ^{15}N-nitrite 1, 10 and 100 µmol/l (or vehicle) and nitrate 300 µmol/l (or vehicle) were added to each sample. After 30 min of incubation, the rings were washed three times with PBS solution and frozen at −70 °C for later analysis.

Ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) analysis was used to measure ^{15}N-nitrite concentrations. The aortic rings were weighed, powdered and solubilized in water. Then ^{15}N-nitrite was converted to ^{15}N-2,3-naphthotriazole (^{15}N-NAT) by reaction with 2,3-diaminonaphthalene (DAN) [23]. Briefly, 22 µL of DAN 0.5 mmol/l were diluted in HCl 0.5 mol/l and incubated with 200 µL of the samples at room temperature. The reaction was stopped after 10 min by adding 12 µL of NaOH 2.4 mol/l, and then diluted with 206 µl of the CH_3CN and centrifuged at 10,500*g* for 5 min.

UPLC-MS/MS analysis was carried out using a Waters ACQUITY UPLC *H-Class* system coupled to the Xevo® TQ-S tandem quadrupole (Waters Corporation, Milford, MA, USA) mass spectrometer with a Z-spray source operating in the positive mode. Five µL of the sample was injected into an Ascentis Express C18 column (100×4.6 mm i.d.; 2.7 µm particle size) from SUPELCO Analytical. The mobile phase used for gradient elution consisted of MilliQ water with 0.1% formic acid as system A and CH_3CN with 0.1% formic acid as system B. The flow rate was 0.5 ml/min. The gradient elution program started with 40% B, raised B to 95% in the following 4.0 min, remained at 95% B for 3 min, and returned to the initial condition (40% B) within the following 5 min. The source and operating parameters were optimized as: capillary voltage =3.2 kV, cone voltage =40 V, source offset =60 V, Z-spray source temperature =150 °C, desolvation temperature (N_2) =300 °C, desolvation gas flow =600 L h^{-1}. Argon was used as the collision gas at a flow of 0.15 mL min^{-1}.

Quantification of ^{15}N-2,3-naphthotriazole (^{15}N-NAT) was carried out by UPLC-MS/MS in the multiple reaction monitoring (MRM) mode with the powdered aortic rings. The reference standard solutions were prepared at appropriate dilutions of the sodium nitrite stock solutions (2.2 µmol/l) with water were reacted with DAN/HCl and stopped with NaOH and diluted with ACN. The final concentration obtained were (nmol/l): 1000, 500, 250, 125, 62.5, 31.2, 15.6 and 7.8, respectively.

Table 1
Ion transition, instrument settings and retention time for ^{15}N-NAT.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	DP[a] (V)	CE[b] (eV)	RT[c] (min)
^{15}N-NAT	171.1	115.3	30	20	3.66

[a] DP = Declustering Potential.
[b] CE = Collision Energy (Ar was used as collision gas).
[c] RT = Retention Time.

The Xevo TQ-S was tuned so that the precursor and product ions were resolved with a peak width at half height of 0.5 Da. The MRM transition, along with the collision energy for the method is shown in Table 1. Triplicate injections were made for standard solution and samples. The calibration curve obtained in MRM mode was used for quantification of ^{15}N-NAT and the concentrations in the samples were expressed in µmol/l. The data was acquired and processed using TargetLynx™ Application Manager software (Waters, Corporation).

2.7. Assessment of the effects of nitrate on aortic reactivity in response to cumulative concentrations of a NO-donor compound

The vasorelaxing effects of sodium nitrite are mostly attributable to the vasorelaxing effects of NO generated from nitrite [24]. Given that sodium nitrate attenuated the vascular responses to increasing concentrations of sodium nitrite (as shown in Results), we wanted to rule out the possibility that sodium nitrate impairs NO signaling pathway and decreases the vascular responses to a NO-donor drug. To test this possibility, we used the same methods described above (in Section 2.2). We examined the aortic relaxing responses to DETANONOate at 10^{-10} to 10^{-5} mol/l cumulative concentrations [25] in aortas incubated with sodium nitrate 300 µmol/l or vehicle 30 min before precontraction with phenylephrine and constructing DETANONOate concentration-response curves.

2.8. Determination of the effects of sodium nitrate on XOR-mediated nitrite reductase activity

To examine the possibility that nitrate attenuates the vascular and blood pressure responses to nitrite by competing with nitrite for XOR, resulting in impaired XOR-mediated nitrite reductase activity, we carried out *in vitro* experiments using XOR. Nitrite reductase activity assessed with basis on NO production measured by chemiluminescence (Sievers Model 280 NO analyzer). Briefly, XOR 100 mU/ml was injected into a purge vessel containing sodium nitrite 1 mmol/l, reduced β-Nicotinamide adenine dinucleotide phosphate (NADPH) 1 mmol/l and β-Nicotinamide adenine dinucleotide reduced (NADH) 1 mmol/l in phosphate buffer 1 mmol/l, pH 7.4, purged with nitrogen in line with the gas-phase chemiluminescence NO analyzer [26]. To examine whether nitrate inhibits XOR-mediated nitrite reductase activity, we examined the effect of nitrate 30 mmol/l (or vehicle) on NO production when nitrite was added to the purge vessel 5 min before adding XOR to the purge vessel. The production of NO was measured for 20 min.

To further validate our results, we added the selective XOR inhibitor febuxostat 50 nmol/l to inhibit XOR-mediated NO production from nitrite in some experiments. Moreover, in some experiments, sodium nitrate 30 mmol/l was added to the purge vessel approximately 20 min after XOR-mediated nitrite reduction reaction was started in order to examine whether nitrate inhibits this reaction.

Finally, we carried out a series of experiments using the same conditions described above (NADPH 1 mmol/l, NADH 1 mmol/l and XOR 125 mU/2 ml in phosphate buffer 100 mmol/l) to examine the inhibitory effects of sodium nitrate 15 mmol/l (or vehicle) on XOR mediated nitrite reduction to NO when nitrite was used at a wide range

of concentrations (from 15 μmol/l to 150 mmol/l), so that we could build up a Michaelis Menten curve. The rate of NO formation from nitrite was measured for two min after individual experiments for each nitrite concentration. The rate of NO formation *versus* nitrite concentration was plotted.

2.8.1. Statistical analysis

The results are expressed as means ± S.E.M. The comparisons between groups were assessed by unpaired *t* test or by one-way and two-way analysis of variance (ANOVA), and using Dunnett's and Bonferroni post test where appropriated. A probability value < 0.05 was considered significant.

3. Results

3.1. Sodium nitrate impairs the vasorelaxing effects of sodium nitrite in both conductance and resistance vessels

The basic hypothesis of this study was that sodium nitrate could attenuate the vascular responses to sodium nitrite. The experiments carried out using aortic ring preparations showed that pre-incubation of aortic rings with sodium nitrate 300 μmol/l shifted the concentration-response curve in response to sodium nitrite to the right, with significant decrease in pD2 and in the maximum response (pD2=4.25 ± 0.15 and Emax=91.5 ± 5.0 with nitrate 300 μmol/l, and pD2=4.73 ± 0.08 and Emax=105.3 ± 1.9 with vehicle; both $P < 0.05$; Fig. 1a, b, and c, respectively). While incubation with sodium nitrate 200 μmol/l tended to exert similar effects, the differences were not statistically significant ($P > 0.05$; Fig. 1a, b, and c). Importantly, there was no significant effects of pre-incubation of aortic rings with sodium nitrate 200 μmol/l or 300 μmol/l on baseline aortic tone compared with control conditions (1.31 ± 0.12 g in controls *versus* 1.52 ± 0.14 g and 1.40 ± 0.16 g with sodium nitrate 200 μmol/l and 300 μmol/l, respectively), thus showing no direct effects of sodium nitrate.

To further validate our findings using aortic rings, we tested the same hypothesis in mesenteric arterial bed perfusion preparation, so that we would be testing this hypothesis using a vascular associated with increased resistance to blood flow and relevant to blood pressure regulation. Interestingly, we found that nitrite caused concentration-dependent decreases in perfusion pressure of mesenteric arterial bed contracted by phenylephrine (Fig. 2; $P < 0.05$). This effects was attenuated when sodium nitrate 300 μmol/l was added to the perfusion solution, particularly when sodium nitrite was infused at 0.1 and 1.0 μmol/l concentrations (Fig. 2; both $P < 0.05$). The two-way ANOVA showed a P value of 0.094 when all nitrite concentrations were taken into consideration. However, P value was 0.040 if the highest nitrite concentration is not taken into consideration. Together, our findings in conductance and resistance vessels suggest that nitrate attenuates the vascular responses to nitrite.

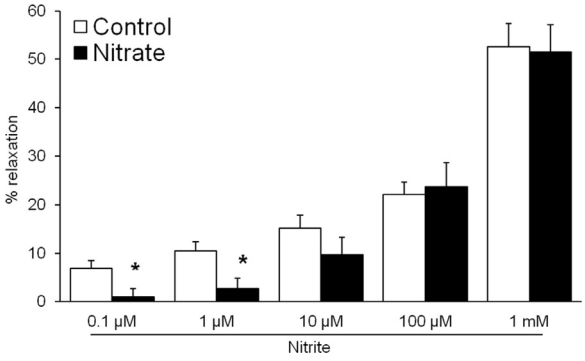


Fig. 2. ***Sodium nitrate impairs the vasorelaxing effects of sodium nitrite in the mesenteric arterial bed***. Nitrite decreased the perfusion pressure of mesenteric arterial bed contracted by phenylephrine in a concentration-dependent manner. Adding sodium nitrate 300 μmol/l to the perfusion solution attenuates this effect. The figure shows the % relaxation (% decrease in perfusion pressure) induced by increasing concentrations of sodium nitrite (from 0.1 μmol/l to 1 mmol/l) to the perfusion solution (Control), which is attenuated in the presence of sodium nitrate 300 μmol/l. Data are shown as mean ± S.E. M. (n=7–8/group). *$P < 0.05$ *versus* Control.

3.2. Sodium nitrate attenuates the in vivo blood pressure responses to sodium nitrite in freely moving rats

Blood pressure responses to the acute administration of sodium nitrite were examined in freely moving rats after the administration of sodium nitrate (or vehicle) to test the possibility that the vascular effects described above would translate into significant interference of nitrate on the blood pressure responses to nitrite. To maximize the probability of finding effects, the rats were made hypertensive by the acute administration of L-NAME. Baseline MAP were 98.2 ± 3.2 and 96.0 ± 2.4 mmHg in vehicle and in nitrate groups, respectively ($P > 0.05$). In addition, the MAP reached 141.5 ± 1.8 mmHg and 141.8 ± 2.2 mmHg, respectively, after L-NAME in vehicle and in nitrate groups rats ($P > 0.05$). While we found that sodium nitrite induced dose-dependent reductions in MAP (Fig. 3a and b; $P < 0.05$), sodium nitrate (40 μmol/kg) attenuated this effect, particularly when the highest sodium nitrite dose was administered ($P < 0.001$, Fig. 3a and b).

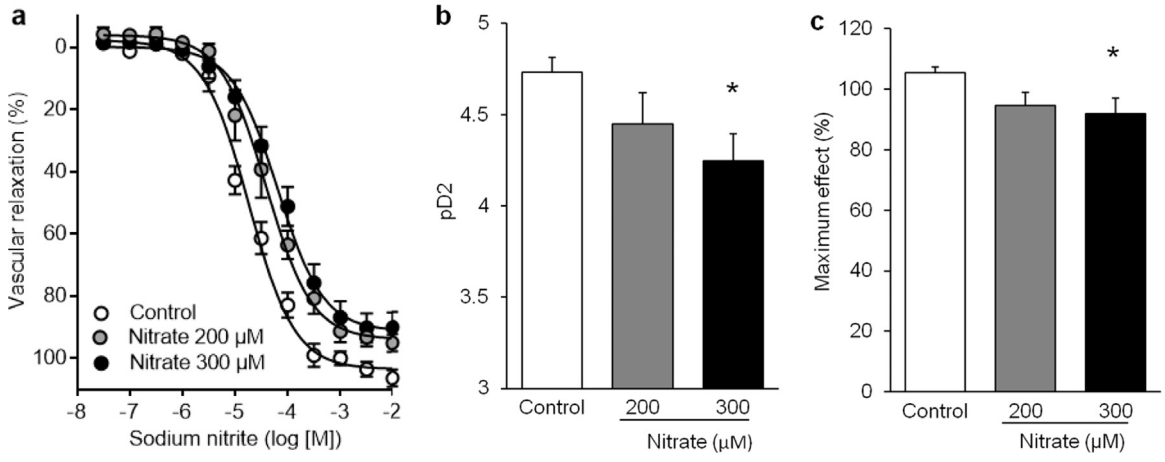


Fig. 1. ***Sodium nitrate impairs the aortic vasorelaxing effects of sodium nitrite***. Incubation of aortic rings with sodium nitrate 300 μmol/l shifted the concentration-response curve in response to sodium nitrite to the right, with significant decrease in pD2 (the negative logarithm of the concentration producing half maximum effect) and in the maximum response. Sodium nitrate 200 μM tended to exert similar effects. Panel a shows the relaxation response curves to cumulative concentrations of sodium nitrite under controls conditions (vehicle) and in the presence of sodium nitrate 200 and 300 μmol/l. Panel b shows pD2 values. Panel c shows the maximum effect. Data are shown as mean ± S.E.M. (n=4–8/group).* $P < 0.05$ *versus* the Control group.

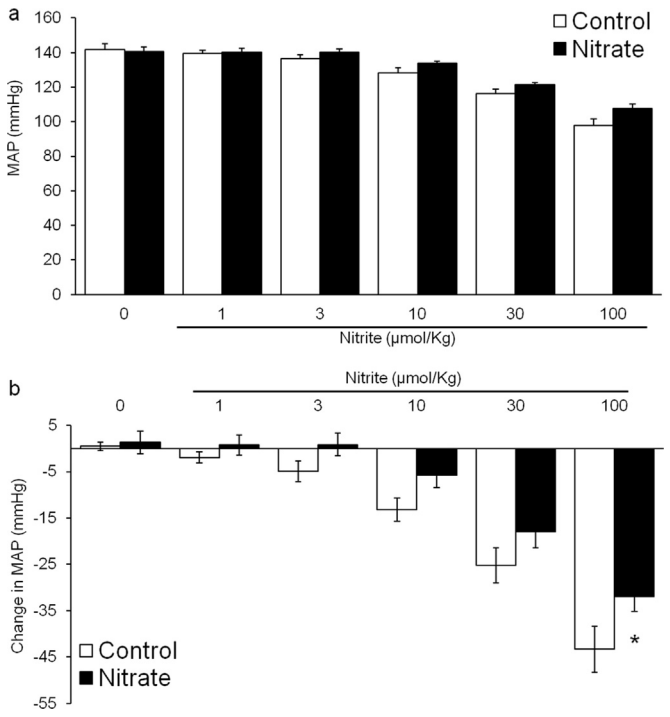


Fig. 3. ***Sodium nitrate attenuates the in vivo mean arterial blood pressure (MAP) responses to sodium nitrite in freely moving rats***. The rats were made hypertensive by the acute administration of L-NAME (100 mg/kg, by gavage). Forty min later, the rats received nitrate (40 μmol/kg) or saline (controls) intravenously. Ten minutes later, increasing doses of sodium nitrite (1, 3, 10, 30 and 100 μmol/Kg) were infused intravenously every ten min. Panel a shows the MAP after each dose of nitrite in rats that received saline (open bars) or nitrate (closed bars). Panel b shows the changes in MAP after each dose of nitrite in rats that received saline (open bars) or nitrate (closed bars). Data are shown as mean ± S.E.M. (n =5–6/group). P < 0.001 for two-way ANOVA. *P < 0.05 *versus* respective Control group.

These findings are consistent with the idea that nitrate attenuates the vascular and blood pressure responses to nitrite.

The plasma concentrations of both nitrite and nitrate were determined in rats receiving both anions during blood pressure response experiments, so that we could provide at least a general view of the concentrations of both anions while interacting to affect blood pressure. While plasma nitrite concentrations increased from less than 0.7 μmol/l to approximately 40 μmol/l in nitrite-treated rats (P < 0.05; Fig. 4a), pretreatment with sodium nitrate did not affect this change in plasma nitrite concentrations (P > 0.05; Fig. 4a). Conversely, while plasma nitrate concentrations increased from 42 μmol/l to approximately 315 μmol/l in rats receiving nitrite infusions after vehicle administration (P < 0.05; Fig. 4b), this variable increased to approximately 414 μmol/l in rats receiving nitrite infusions after nitrate administration (P < 0.05 *versus* control group; Fig. 4b).

3.3. Sodium nitrate does not affect the vascular influx and accumulation of nitrite labeled with stable isotope (^{15}N-nitrite)

Because the attenuation of the vascular responses to sodium nitrite by sodium nitrate could not be explained by impaired NO-relaxing effects, experiments were carefully designed to explore the possibility that nitrate could compete with nitrite by mechanisms involving the nitrite influx into cells. Therefore we examined this possibility by carrying out experiments to measure the accumulation of ^{15}N-nitrite by aortic rings in the presence of sodium nitrate 300 μmol/l. While incubation with ^{15}N-nitrite 1, 10, and 100 μmol/l increased nitrite concentrations in a concentration-dependent manner (to 0.75 ± 0.10, 1.65 ± 0.17, and 16.59 ± 4.71 μmol/l, respectively; Fig. 5; P < 0.05), the presence of sodium nitrate 300 μmol/l did not affect nitrite accumulation (Fig. 5; P > 0.05). These results strongly suggest lack of significant interference of nitrate on nitrite influx, and therefore the attenuation of the vascular responses to sodium nitrite by sodium nitrate are probably not explained by competitive mechanisms related to crossing membrane barriers.

*3.4. Sodium nitrate does not affect the vasorelaxing effects of the **NO donor** DETANONOate*

Sodium nitrate attenuated the vasorelaxing responses to sodium nitrite, which exerts its effects mostly by generating the vasorelaxing mediator NO [24]. Therefore, this effect could be attributable attenuation of NO-mediated relaxation by nitrate. To test this possibility, the aortic relaxing responses to DETANONOate were studied during incubation of aortic rings with nitrate and with vehicle. While DETANONOate caused concentration-dependent vasorelaxing effects with pD2=6.48 ± 0.06 and Emax=87.08 ± 2.64, pre-incubation nitrate 300 μmol/l did not affect this response (Fig. 6a, b, c; P > 0.05), thus suggesting that nitrate does not affect NO-dependent vascular effects.

3.5. Nitrite reductase activity of XOR is attenuated by nitrate

Given that XOR is actively involved in the generation of nitrite from nitrate and NO from nitrite, we designed experiments to test the possibility that nitrate could compete with nitrite for XOR, and therefore nitrate could attenuate NO formation from nitrite by competitively inhibiting XOR-mediated nitrite reductase activity. Our *in vitro* experiments using XOR showed that nitrate 30 mmol/l attenuated XOR-mediated NO formation from nitrite 1 mmol/l by approximately 60% (from 256.4 ± 22.5 to 104.9 ± 8.4 pmol/min; Fig. 7; P < 0.05; n=3). Interestingly, the selective XOR inhibitor febuxostat (50 nmol/l) further inhibited XOR-mediated NO production from nitrite (Fig. 7b). Moreover, we found that adding sodium nitrate 30 mmol/l to the reaction solution containing XOR and nitrite immediately inhibited NO formation from nitrite (Fig. 7b).

Next, we carried out experiments to carefully examine the inhibitory effects of sodium nitrate 15 mmol/l (or vehicle) on XOR-mediated nitrite reductase activity. We measured the rate of NO formation *versus* nitrite concentrations, and we found that nitrate significantly shifted the Michaelis Menten saturation curve to the right (Fig. 8; P < 0.05). While nitrate did not change the maximum rate (V_{max}), the Michaelis constant (K_m, which corresponds to the nitrite concentrations at which the reaction rate is half of V_{max})increased by almost 3-fold (from 9.3 ± 2.5 to 27.1 ± 6.8 μmol/min/mg, Fig. 8, P < 0.05). Together, these results show that nitrate can inhibit XOR-mediated NO production from nitrite, and this mechanism could explain our functional findings.

4. Discussion

This study shows functional *in vivo* and *in vitro* evidence that nitrate attenuates the vascular and blood pressure responses to nitrite, probably as a result of interference with XOR-mediated nitrite reductase activity. This is the first study to provide evidence suggesting that although nitrate may serve as a reservoir of NO, it may prevent excessive or too fast NO formation from nitrite.

NO is oxidized to form nitrite and nitrate, and both anions recycle back to NO through a nitrate-nitrite-NO pathway that may complement endogenous NO synthase-dependent formation from L-arginine [5,27]. While the importance of nitrite as a major vascular storage pool and source of NO has recently been valued [28–30], the mechanisms explaining its effects are not entirely known. However, the fact that both anions must cross membrane barriers [9] before they are converted into NO has never being explored as a possible interaction point between both anions. Giving support to this idea, plasma nitrite concentrations are usually below 0.5 μmol/l, whereas plasma nitrate

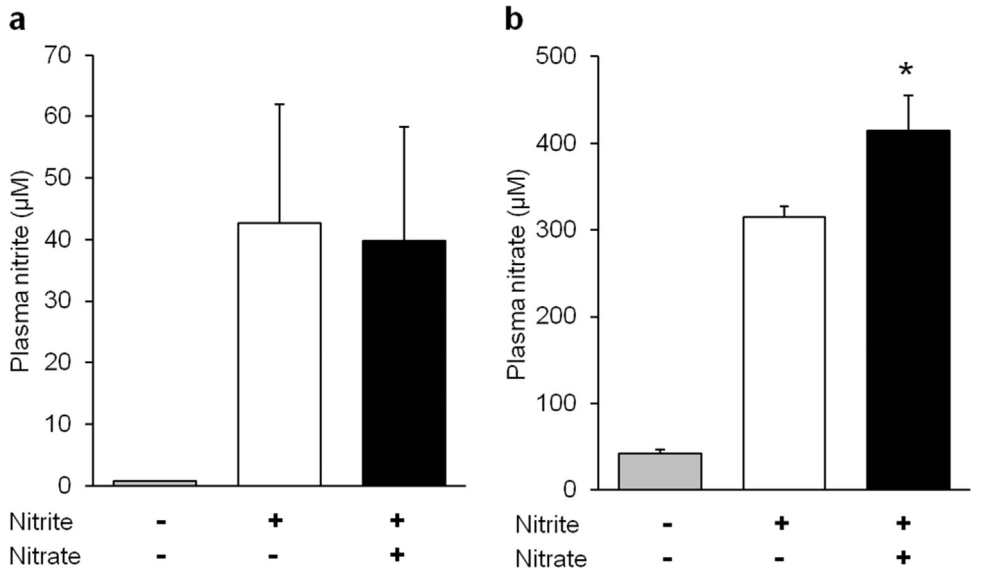


Fig. 4. Plasma nitrite and nitrate concentrations in rats after intravenous administration. The rats received L-NAME (100 mg/kg, by gavage) followed 40 min later by sodium nitrate (40 μmol/kg) or saline intravenously, and then doses of sodium nitrite (1, 3, 10, 30 and 100 μmol/Kg) intravenously, every ten min. Panels a and b show nitrite and nitrate concentrations, respectively, at the end of the experiments. Plasma nitrite and nitrate were also measured in some untreated rats. Data are shown as mean ± S.E. M. (n=5–6/group). *P < 0.05 comparison between nitrate and saline treated rats.

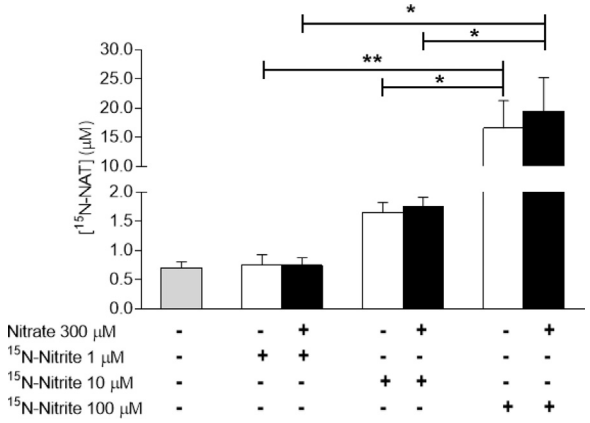


Fig. 5. Sodium nitrate does not affect the vascular influx and accumulation of nitrite labeled with stable isotope (^{15}N-nitrite). Aortic rings were incubated with ^{15}N-nitrite 1, 10 and 100 μmol/l (or vehicle) and nitrate 300 μmol/l (or vehicle) for 30 min, and then washed three times with PBS. The vascular concentrations of ^{15}N-2,3-naphthotriazole (^{15}N-NAT) were measured by Ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) after ^{15}N-nitrite was converted to ^{15}N-NAT. Data are shown as mean ± S.E.M.(n=5–12/group). * P < 0.05** P < 0.01.

concentrations are usually two orders of magnitude higher [21], and therefore the possibility that both anions could compete for anion channels or other passive diffusion mechanisms seems plausible [31,32].

For the reason explained above, we carried out a series of functional experiments to define whether there nitrate could at least attenuate the vascular responses to nitrite. Interestingly, we found that nitrate attenuated the vascular responses to increasing concentrations of nitrite, both in conductance (aorta) and in resistance (mesenteric vascular bed) vessels. Moreover, we carried out *in vivo* experiments to further validate this inhibitory interaction between anions, and the blood pressure responses to increasing doses of nitrite were again attenuated by nitrate. However, although nitrate attenuated the vascular and blood pressure responses to nitrite, we found that this effect of nitrate probably does not result of a competitive mechanism involving impaired nitrite influx into vascular tissue. Indeed, we measured the accumulation of nitrite by aortic rings in the presence of sodium nitrate 300 μmol/l using nitrite labeled with a stable isotope (^{15}N-nitrite). This approach was chosen to allow the assessment of small increases in vascular concentrations of nitrite, which could be limited by baseline nitrite concentrations causing signal-to-noise issues in measuring such increases. Our results clearly show similar increases in vascular ^{15}N-nitrite concentrations in aortas incubated with this labeled nitrite in the presence or in the absence of nitrate, thus providing strong evidence that the functional interaction between nitrate and nitrite are not explained by differences in nitrite influx into tissues.

Next, we examined whether nitrate-induced attenuation of the vascular responses to nitrite could be explained by impaired vasorelaxing responses to NO derived from nitrite [24]. In fact, the fall in the blood pressure [33] and the vasorelaxing responses [34] to sodium nitrite in rats were strongly attenuated by a NO scavenger. Therefore, if nitrate impaired vascular NO signaling pathways, we would also expect the responses to a NO donor drug to be affected. Interestingly, we found virtually identical aortic relaxing responses to DETANONOate [25] after incubation with nitrate 300 μmol/l or vehicle. This finding strongly suggested no relevant effect of nitrate on NO signaling and vascular responses.

Finally, the possibility that NO generation from nitrite could be affected by nitrate was tested in this study. This is very important because apparently most of NO generation from nitrite takes place in the tissues [30], and this mechanism is implicated in the responses to circulating nitrite [5]. Among other enzymes with nitrite-reductase activity, special attention should be given to XOR because this enzyme generates NO from both nitrite and nitrate anions [11,14–16]. Therefore, we tested the possibility that nitrate could interfere with XOR-mediated NO generation from nitrite. Interestingly, we found that nitrate significantly decreased NO formation from nitrite by XOR-mediated mechanisms and increased the Michaelis constant by 3-fold. To our knowledge, this is the first report to show that nitrate may interfere with NO production from nitrite.

While XOR reduces both nitrate and nitrite anions to NO, there are some relevant differences between both XOR-catalyzed reactions [16]. As previously determined under hypoxic conditions, nitrate reduction to NO followed Michaelis-Menten kinetics (with K_m and V_{max} values of 0.29 ± 0.6 mmol/l and $9.7 \pm 0.3 \times 10^{-1}$ mol/min/mg, respectively), whereas apparent corresponding values for nitrite reduction to NO were 22.9 ± 8.1 mmol/l and $3.73 \pm 0.72 \times 10^{-1}$ mol/min/mg, respec-

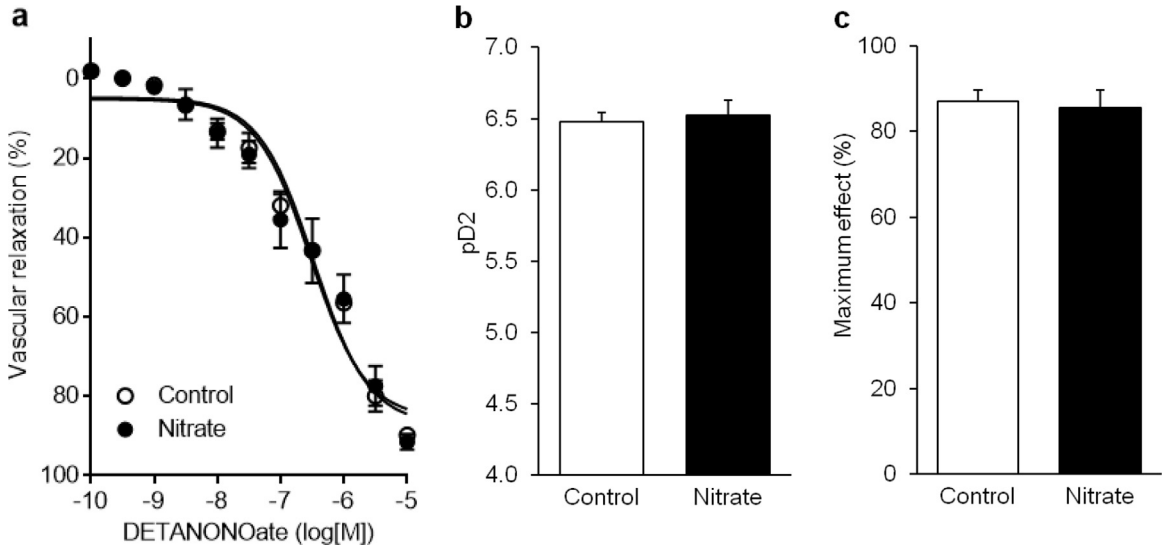


Fig. 6. ***Sodium nitrate does not affect the aortic vasorelaxing effects of the NO-donor diethylenetriamine NONOate (DETANONOate)***. Incubation of aortic rings with sodium nitrate 300 μmol/l did not affect the aortic relaxing responses to DETANONOate studied at 10^{-10}–10^{-5} mol/l cumulative concentrations. Panel a shows virtually identical relaxation response curves to cumulative concentrations of DETANONOate under controls conditions (vehicle) and in the presence of sodium nitrate 300 μmol/l. Panel b shows pD2 values. Panel c shows the maximum effect. Data are shown as mean ± S.E.M. (n=6/group).

tively [16]. Although never tested before, our results showing that nitrate decreases XOR-mediated NO formation from nitrite are consistent with these previous findings [16]. It should be taken into consideration that more complexity should be expected because NO derived from nitrite or nitrate may inhibit XOR [37,38], and therefore the regulation of nitrite-reductase *in vivo* XOR activity may be more complicated than suggested here.

Our chemiluminescence results suggest that the inhibition of XOR-mediated nitrite reductase activity by nitrate is probably competitive and reversible. This finding may explain, at least in part, how nitrate shifts to the right the aortic concentration-dependent curve responses to nitrite, as XO is involved in vascular relaxation induced by nitrite [39]. However, it is unclear why nitrite at mM concentrations does not cause total relaxation in the presence of nitrate (as shown in Fig. 1), thus challenging the suggestion of a competitive mechanism. While we have no precise explanation for this finding, it is possible that other endogenous factors may affect XOR activity by interfering with its molybdenum center or phosphorylation or other allosteric effect [36,40]. Moreover, it is possible that some differences between *in vivo* and *in vitro* results are attributable to other enzymes involved in the reduction of nitrite to NO, including mitochondrial enzymes, sGC or other heme-containing enzymes expressed in the vasculature [34].

We used the Lineweaver-Burk equation to assess how nitrate 0.3 mmol/l inhibits NO production from nitrite 0.1 mol/l, and this calculation resulted in only 4% inhibition for the *ex vivo* conditions shown in Fig. 1. While this effect may not entirely explain our functional *in vivo* or *ex vivo* results, it should be clear that our *in vitro* conditions may not reproduce tissue biology completely. A variety of factors could affect the Km or velocity of XOR-mediated reactions using nitrite as a substrate, such as absence of oxygen [35], excess of NADH [36], absence of xanthine [36], and the activity of other enzymes that could scavenge reactive oxygen species [36]. Moreover, the

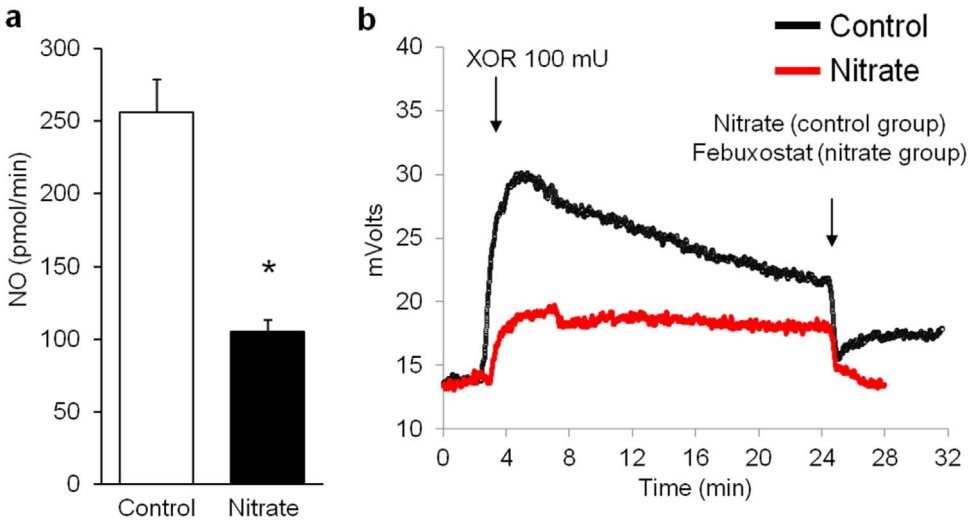
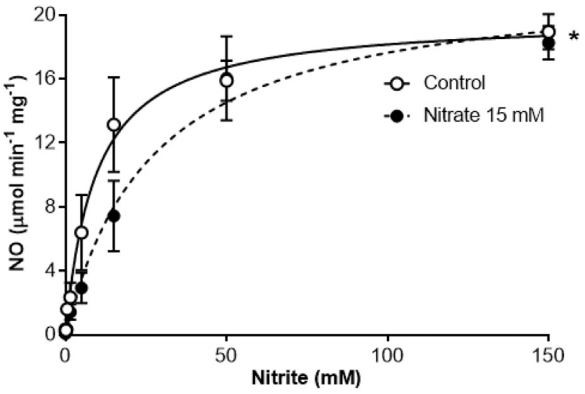


Fig. 7. Nitrate inhibits XOR mediated nitrite reductase activity. Panel a shows the rate of XOR-mediated NO formation from 1 mmol/l sodium nitrite in the presence of nitrate 30 mmol/l, which is approximately 60% lower than that found with vehicle (Control). Panel b shows representative tracings of NO formation from nitrite. The black tracing (Control group) shows that XOR activates NO formation from nitrite. Adding nitrate 30 mmol/l approximately 20 min after the reaction started immediately inhibits NO production. The red tracing shows that the presence of nitrate 30 mmol/l at baseline conditions (Nitrate group) attenuates XOR-mediated NO formation from nitrite by approximately 60%. Adding the selective XOR inhibitor febuxostat (50 nmol/l) to the reaction further inhibited XOR-mediated NO production from nitrite. Data are shown as mean ± S.E.M. (n=3). * $P < 0.01$ *versus* Control.



	Control	Nitrate	p
Vmax (µmol/min/mg)	19.8 ± 1.5	22.4 ± 2.0	ns
Km (mM)	9.3 ± 2.5	27.1 ± 6.8	<0.05

Fig. 8. Michaelis-Menten saturation curve to show that sodium nitrate inhibits XOR-mediated nitrite reductase activity and NO production. The rate of NO formation *versus* nitrite concentrations are shown for Control (vehicle) and in the presence of sodium nitrate 15 mmol/l. Nitrate significantly shifted the Michaelis Menten saturation curve to the right. The maximum rate (V_{max}) was not affected by nitrate, whereas the Michaelis constant (K_m)increased by almost 3-fold ($P < 0.05$). Data are shown as mean ± S.E.M. (n=3–4).* $P < 0.01$ for between curves comparison.

concentration of XOR we utilized was probably in excess as compared with those found in rats [11,37]. Therefore, the extent of nitrate-induced inhibition or XOR-mediated nitrite reductase activity in the tissues could be significantly different from that found in *in vitro* studies. Further studies are required to define the mechanism explaining how nitrate decreases the Emax in response to nitrite.

Our findings may have important implications beyond the vascular and blood pressure responses to nitrite. While it is obvious that nitrate may act as a reservoir of NO that is less promptly reduced to NO than nitrite, our results suggest that nitrate may exert an additional role. Nitrate may prevent excessive NO generation from nitrite, and this may be particularly important under conditions associated with increased XOR expression or activity. For example, while it is possible that increased vascular XOR activity contributes to the antihypertensive effects of nitrite in hypertension [11,12,37], it is possible that exogenously administered nitrite is oxidized to nitrate, which in turn attenuates XOR-mediated NO production. This reaction would decrease excessive amounts of nitrite from being reduced to NO. It remains to be proved whether this notion is applicable to other conditions associated with increased circulating nitrate concentrations. This could well be the case of sepsis [41,42].

In conclusion, our results provide functional evidence that nitrate attenuates the vascular and blood pressure responses to nitrite. This effect is probably explained by the inhibitory effect of nitrate on XOR-mediated nitrite reductase activity promoting NO formation from nitrite.

Acknowledgments

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