Paradoxical Normoxia-Dependent Selective Actions of Inorganic Nitrite in Human Muscular Conduit Arteries, and Related Selective Actions on Central Blood Pressures

Running title: Omar et al.; Nitrite - Selective Dilator of Conduit Arteries

Sami A Omar, MRCP, MBBS1; Henry Fok, MRCP, MBBS1; Katharina D Tilgner, BSc, MSc1; Ashok Nair, FRCA, MSc, MBBS12; Joanne Hunt, PhD1; Benyu Jiang, PhD1; Paul Taylor, PhD1; Phil Chowienczyk, FRCP1; Andrew Webb, FRCP, PhD1

1King’s College London British Heart Foundation Centre, Cardiovascular Division, Dept of Clinical Pharmacology, London, United Kingdom; 2Biomedical Research Centre, Guy’s & St Thomas’ NHS Foundation Trust, London, United Kingdom

Address for Correspondence:
Andrew Webb, FRCP, PhD
Department of Clinical Pharmacology, 4th Floor
North Wing, St. Thomas’ Hospital
London SE1 7EH, United Kingdom
Tel: +44 2071884602.
Fax: +44 2071885116.
E-mail: andrew.1.webb@kcl.ac.uk

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Abstract

**Background**—Inorganic nitrite dilates small resistance arterioles, via hypoxia-facilitated reduction to vasodilating nitric oxide (NO). Nitrite’s effects in human conduit arteries have not been investigated. In contrast to nitrite, organic nitrates are established selective dilators of conduit arteries.

**Methods and Results**—We examined effects of local and systemic administration of sodium nitrite on the radial artery (a muscular conduit artery), forearm resistance vessels (forearm blood flow) and systemic hemodynamics in healthy male volunteers (n=43). Intra-brachial sodium nitrite (8.7 μmol/min) increased radial artery diameter by 28.0% (25.7, 40.1), median (25th, 75th percentiles), P<0.001. Nitrite (0.087-87 μmol/min) displayed similar conduit artery selectivity as glyceryl trinitrate (0.013-4.4 nmol/min), over resistance arterioles. Nitrite dose-dependently increased local cGMP production from the dose of 2.6 μmol/min, by 1.1 pmol/min/100ml tissue (95% CI 0.5 to 1.8). Nitrite-induced radial artery dilation was enhanced by administration of acetazolamide (oral or intra-arterial) and oral raloxifène (P=0.0248, P<0.0001 and P=0.0006, respectively) but was inhibited under hypoxia (P<0.0001) and hyperoxia (P=0.0006) compared to normoxia. Systemic intravenous administration of sodium nitrite (8.7 μmol/min) dilated the radial artery by 10.7% (95% CI 6.8 to 14.7) and reduced central systolic BP by 11.6 mmHg (95% CI 2.4 to 20.7), augmentation index and pulse wave velocity, without changing peripheral BP.

**Conclusions**—Nitrite selectively dilates conduit arteries at supra-physiological and near-physiological concentrations via a normoxia-dependent mechanism, which is associated with cGMP production, and is enhanced by acetazolamide and raloxifene. Nitrite’s selective central BP-lowering effects have therapeutic potential to reduce cardiovascular events.

**Key words:** nitrite, conduit arteries, carbonic anhydrase, aldehyde oxidase, central blood pressure, nitric oxide, nitroglycerin, reactive oxygen species, hypoxia, vasodilation
Introduction

The endogenous anion, inorganic nitrite (NO₂⁻) is a hypoxia-dependent dilator of small resistance arterioles, via its reduction to vasodilating nitric oxide (NO).¹ A local hypoxic environment is created in small resistance arterioles due to oxygen extraction by skeletal muscle, causing intravascular oxygen levels to fall to around the P50 of hemoglobin (the oxygen concentration at which half the heme is saturated with oxygen) facilitating near-maximal conversion rates of nitrite to NO by deoxyhemoglobin and hence vasodilation of resistance arterioles, as measured by an increase in forearm blood flow (FBF).²

Responses to nitrite are further enhanced under hypoxic conditions in resistance arterioles (FBF).³,⁴ Indeed, all the nitrite reductases identified to date, display selective activity under conditions of hypoxia or ischemia;⁵ including the other globins (myoglobin, cytoglobin and neuroglobin), xanthine oxidase (XO), endothelial nitric oxide synthase (eNOS), and aldehyde oxidase (AO).⁶-⁸ Conversely, oxygenated hemoglobin and myoglobin are avid scavengers of nitrite-derived NO, suggesting an intricate balance exists between production and consumption.⁷-⁹

Most antihypertensive vasodilators selectively dilate small resistance arterioles,¹⁰ (mainly reducing peripheral blood pressure). Notable exceptions are the organic nitrates, e.g. glyceryl trinitrate (GTN) a selective dilator of muscular conduit arteries,¹⁰-¹² the accepted mechanism by which GTN selectively lowers central blood pressure.¹³ We therefore hypothesized that inorganic nitrite is a selective hypoxia-dependent resistance arteriolar dilator, with minimal effect on conduit arteries. We tested nitrite’s vascular/hemodynamic mechanisms and selectivity in normoxia, hypoxia and hyperoxia, the effect of inhibition of AO with raloxifene (to determine the net effect of inhibition of AO-derived reactive oxygen species (ROS),¹⁴ versus AO-dependent nitrite reduction),¹⁵-¹⁷ and the effect of acetazolamide, which may enhance the nitrite
Methods

Volunteers

The studies were approved by the St Thomas’ Research Ethics Committee (South East London REC2) and NRES Committee London – Westminster. Subjects were healthy males recruited from the community. Informed written consent was obtained from all the participants.

Study Protocols and Vascular/Hemodynamic Measurements

Participants were instructed to fast overnight and asked to avoid ingesting nitrate-rich foods for 24 hours prior to the study. The effects of locally administered intra-brachial sodium nitrite or GTN, or systemically administered intravenous sodium nitrite, on radial artery diameter, imaged using 2-dimensional ultrasound, resistance arteriolar flow (FBF) measured using venous occlusion plethysmography, systemic hemodynamic parameters, and plasma nitrite concentrations from serial venous blood samples were assessed in a series of 6 studies (for full methodological details please refer to the online-only Data Supplement):

- **Study 1**: Single-visit assessment of fixed dose i.a. sodium nitrite (8.7 μmol/min): ipsilateral radial artery.

- **Study 2**: Single-visit (parallel-groups), assessing ipsilateral radial artery responses to:
  - (a) i.a. sodium nitrite (0.087-87 μmol/min)
  - (b) i.a. GTN (0.013-4.4 nmol/min).

- **Study 3**: Effect of (i) oral acetazolamide (500 mg), and (ii) raloxifene (120 mg) versus (iii) placebo (standard lactose tablet) administered 2h before i.a. sodium nitrite (0.087-26 μmol/min) assessing radial artery responses. This single-blind cross-over study used a minimum of 7
days between visits (exceeding 5-times the half life of acetazolamide and raloxifene) to minimise the risk of carryover effects, and a balanced randomized order design of interventions to limit period effects.

- Supplementary in vitro study: effects of acetazolamide and raloxifene on nitrite-induced dilation in rat aorta (see Supplemental Methods).

- **Study 4:** Effect of a co-infusion of i.a. acetazolamide and sodium nitrite on radial artery diameter and FBF. On the first two visits nitrite was infused alone at a fixed dose (2.6 μmol/ml), or co-administered with acetazolamide (0.1 – 3.0 mg/ml), each dose for 7 min with measurements taken at the end of this period, in a single-blind, randomized, balanced cross-over design; minimum 7-day interval between visits. On the 3rd visit i.a acetazolamide was infused alone (0.1 – 3.0 mg/ml).

- **Study 5:** Effect of (i) normoxia (inhaling room air (21%O2)), (ii) hypoxia (12%O2), and (iii) hyperoxia (100%O2) on the dose response to i.a. sodium nitrite (0.087 – 26 μmol/ml) in the radial artery. The study design was single-blind, randomized, balanced cross-over; minimum 7-day interval between each of the 3 visits.

- **Study 6:** Single-visit assessment of fixed dose i.v. sodium nitrite (8.7 μmol/min): contralateral radial artery.

**Data and Statistical Analysis**

The data from the volunteer studies was analysed using the Graph Pad Prism Software, while that from the rat thoracic aorta myography studies was analysed using Stata (Version 11.2). All data are expressed as mean±SEM unless otherwise stated. Data were compared by repeated-measures ANOVA (2-tailed) with Dunnett’s post test for comparison with baseline and Bonferroni post test for comparison with the control group. P values <0.05 were considered
statistically significant, unless Bonferroni corrections for multiple comparisons were required, as stated.

Results

Subjects

A total of 43 healthy males participated, of whom 16 took part in more than 1 study. The demographic data for each study are shown in Supplemental Table 1 in the online-only Data Supplement.

Study 1 – Fixed dose intra-arterial sodium nitrite

The intra-arterial infusion of sodium nitrite (8.7 μmol/ min) into the brachial artery resulted in marked dilation of the radial artery (P<0.0001); for example, by 28.0% (25.7, 40.1), median (25th, 75th percentiles), P<0.001, at 60 min compared to baseline saline, Figure 1A. This 28% increase in diameter represents a ~200% (or ~3-fold) increase in radial artery area. The radial artery dilation to nitrite was very marked in one volunteer; however, a significant increase in radial artery diameter was seen irrespective of whether data from this individual was included or excluded. Furthermore, the mean oxygen saturation of hemoglobin was 99% (range 97-100%), i.e. this dilation did not occur under conditions of reduced oxygen tension found in small arterioles, or hypoxia (or ischemia) which have been considered essential for the effects of nitrite via its reduction to NO, but rather occurred under fully oxygenated conditions that are generally considered to inhibit nitrite bioactivation.

As expected, sodium nitrite infusion increased FBF: this was by 1.9 ml/ min/ 100 ml tissue (95% CI 0.56 to 3.25) at 5 min, see Figure 1B, with no further significant increase over 60 min in the infused arm. However, FBF remained unchanged in the control forearm throughout
the 60 min (see Figure 1B). Also, no significant changes in peripheral brachial blood pressure (systolic, SBP, mean arterial, MAP, or diastolic, DBP) were detected during the 60 min infusion of sodium nitrite compared to baseline, i.e. time 0 min (Supplemental Figure 1).

**Study 2 – Dose responses to (a) intra-arterial sodium nitrite and (b) intra-arterial GTN**

In Study 2(a) we examined the effects of intra-brachially administered sodium nitrite across an extensive dose range. This study comprised a low dose series (0.087-2.6 μmol/ min) with subjects returning to complete the higher dose series (8.7, 26 and 87 μmol/ min), with each dose being infused for 25 min, except for the highest dose which was limited to 7 min. The mean baseline radial artery measurements between the first and second visit were similar: 2.32±0.53 mm and 2.50±0.28 mm respectively (mean±SD).

The lowest dose of nitrite (0.087 μmol/ min) resulted in radial artery dilation of 0.06 mm (95% CI 0.003 to 0.12) as shown in Figure 2A. Forearm blood flow increased at a higher dose of nitrite (0.87 μmol/ min) by 0.83 ml/ min/ 100ml tissue (95% CI 0.16 to 1.5) as demonstrated in Figure 2D.

**Figure 3A** demonstrates a clear dose-response to nitrite in terms of radial artery diameter, with nitrite (0.87 μmol/ min) dilating the radial artery by 11.2% (95% CI 2.6 to 19.8), whilst the highest dose of nitrite (87 μmol/ min) resulted in a dilation of 35.0% (95% CI 26.4 to 43.6).

Radial artery measurements were made at 5 min following the initiation of each dose, while FBF was measured at 20 min. However, with nitrite (26 μmol/ min) radial artery measurements were also made at 20 min, and no differences were seen between this time point and 5 min.

Given that 0.9% saline has a pH of ~5.5, we performed a separate series of experiments (Study 2 (aii)) with nitrite in saline, pH-balanced with sodium bicarbonate to pH 7.5±0.13
(mean±SD) and found similar degrees of radial artery dilation: see Online Supplemental Figure 2.

In Study 2(b) GTN (0.013-4.4 nmol/min= 0.003-1 μg/min) resulted in significant radial artery dilation (compared to baseline), with the dose of 0.044 nmol/min, resulting in a dilation of 13.6% (95% CI 2.9 to 22.4). The highest GTN dose (4.4 nmol/min) resulted in a dilation of 33.3% (95% CI 22.5 to 44.1) see Figure 3B.

The selectivity of nitrite and GTN for vasodilating conduit arteries versus small resistance arterioles was compared by plotting the change in radial artery diameter, against the change in FBF at each dose step (see Figure 3C). Nitrite resulted in a gradient of 4.9±0.6 (%/ml/min/100ml), with a strong correlation between conduit artery and resistance arteriole responses, \( r^2 = 0.58, P<0.0001 \). With GTN the gradient was steeper, 5.7±1.3 (%/ml/min/100ml), but with a weaker association than nitrite, \( r^2 = 0.33, P<0.0001 \). This probably reflects greater curvature in the GTN data, and thus a weaker linear association (r). Overall, however, the large/conduit artery selectivity of GTN and nitrite were similar. The effects of i.a. nitrite and GTN on FBF alone are shown in Supplemental Figure 3.

To determine whether nitrite-induced conduit artery dilation was flow-mediated, an analysis of the Doppler US flow images was undertaken. This demonstrated that there was no change in mean radial artery flow (see Supplemental Figure 4) despite the increase in flow in resistance vessels (FBF). This finding is in keeping with a previous study, which demonstrated no change in mean flow - despite dilation of the radial artery, with GTN. 19

No changes in peripheral SBP, DBP or MAP or HR were found following the highest GTN dose, or with nitrite up to the penultimate dose of 26 μmol/min. However, at the highest dose of nitrite (87 μmol/min) for 7 min, BP decreased by ~5.5/14 (11) mmHg (SBP/DBP (MAP) respectively) and heart rate (HR) increased by 19 bpm (P<0.001). The MAP transiently fell by
more than the pre-specified threshold of 15 mmHg in only one subject, at the end of the infusion of the highest dose.

Intra-arterial nitrite (0.087-2.6 μmol/min) increased forearm venous capacitance in the infused arm overall by 45.6 ml/100 ml (95% CI 13.5 to 77.8) compared to the control arm, but not to baseline; see Supplemental Figure 5. GTN increased venous capacitance above baseline, though not when compared to the control arm; however, it should be noted that the sample sizes in these additional assessments were small (n=3).

Systemic methemoglobin levels increased from 1.3±0.1% to 3.5±0.2% with the highest dose of nitrite, associated with a concomitant decrease in hemoglobin oxygen saturations from 98.5±0.3% to 97.0±0.4% (see Supplemental Figure 6). Methemoglobin levels in the infused arm increased to 6.0±1.3%.

**Plasma nitrite, cGMP production and SNOs**

Plasma nitrite concentrations following sodium nitrite infusion in the infused and contralateral arms are shown in Figure 4A: for example, the lowest dose of nitrite (0.087 μmol/min), which resulted in radial artery dilation, was associated with local plasma [nitrite] of 1.05±0.20 μmol/L, which is within the near-physiological range. To determine changes in local cGMP production, we multiplied cGMP plasma concentrations by FBF; units are expressed in picomoles produced per min per 100 ml of tissue. As shown in Figure 4B, nitrite infusion (2.6, 26, 87 μmol/min) increased cGMP production in a dose-dependent manner, by 1.1 pmol/min/100 ml tissue (95% CI 0.5 to 1.8), 2.2 pmol/min/100 ml tissue (95% CI 1.2 to 3.2), and 4.7 pmol/min/100 ml tissue (95% CI 3.3 to 6.2), respectively. However, no systemic changes in cGMP production (in the contralateral arm) were detected. One set of participant’s cGMP data was excluded from the analysis as some cGMP data was missing.
Plasma SNOs in the infused arm were below the level of detection at baseline, and at most of the doses of nitrite infused. However, with nitrite infusion at the highest dose (87 μmol/min), plasma SNOs production was 348±130 pmol/min/100 ml of tissue (data not shown).

**Study 3 – Oral Acetazolamide and Raloxifene**

Since carbonic anhydrase has been described to possess nitrite anhydrase activity,18 we tested its role in human conduit vessels using the carbonic anhydrase inhibitor, acetazolamide. Aldehyde oxidase has been demonstrated to have nitrite reductase activity in homogenised tissues,15 and rabbit and mouse aortic rings, with NO generation and vasorelaxation being inhibited by raloxifene,16, 17 a potent inhibitor of AO.22 However, AO has also been implicated in the generation of ROS,14 and therefore we wished to determine the net effect of inhibition of AO-dependent nitrite reduction versus inhibition of AO-derived ROS.

Oral administration of acetazolamide (500 mg) or raloxifene (120 mg) had no effect on baseline radial artery diameter: 2.689±0.255 mm with placebo, 2.711±0.265 mm with acetazolamide and 2.737±0.257 mm with raloxifene (mean±SD). However, acetazolamide enhanced radial artery dilation to sodium nitrite: reaching the required significance level (P=0.0248), as did raloxifene (P=0.0006), see Figure 5.

In the rat aorta *in vitro*, acetazolamide (10⁻⁷M) caused a reduction in %phenylephrine (PE) preconstriction of the blood vessels by 8.6% (95% CI 3.5 to 13.7). Raloxifene enhanced relaxation to nitrite at all concentrations tested (P=0.0008), see Supplemental Figure 7 in Supplemental Results.

**Study 4 – Intra-arterial Acetazolamide**

Co-infusion of acetazolamide (0.1 - 3.0 mg/ min) with sodium nitrite (2.6 μmol/ min) enhanced nitrite-induced radial artery dilation compared to sodium nitrite alone (P<0.0001), see Figure
For example, acetazolamide (1 mg/min) augmentated nitrite-induced RA dilation by 9.97% (95% CI 0.74 to 19.19) from 15.73 to 25.70%, which equates to a relative increase of ~63%. By contrast, acetazolamide blunted the vasodilatory effect of nitrite in the resistance arterioles (FBF); however, the effect was non-significant (P=0.10), see Figure 6B. The infusion of acetazolamide alone had no effect on FBF; however, it paradoxically diminished (rather than increasing) radial artery diameter (P=0.03).

Acquisition of continuous US imaging of the radial artery following the commencement of nitrite (2.6 μmol/ml) revealed an onset of dilation within 2 minutes (half life 109 s (95% CI 61 to 489)) which was near maximal by the end of 5 minutes (Figure 6C).

**Study 5 – Normoxia, hypoxia, and hyperoxia**

As shown in Figure 7, hypoxia and hyperoxia both inhibited radial artery dilation to nitrite (to a similar extent) compared to normoxia (P<0.0001 and P=0.0006, respectively). Sodium nitrite (8.7 μmol/min) dilated the radial artery by only 19.0±2.6% under hypoxia, compared to 29.1±4.1% under normoxia (absolute difference of 10.1%, 95% CI 1.90 to 18.3). Importantly, baseline radial artery diameter was almost identical under hypoxia, normoxia and hyperoxia: 2.743±0.221 mm, 2.841±0.292 mm, 2.728±0.218 mm (mean ±SD) respectively. In addition, there were no differences in BP or HR between the conditions from baseline to the end of the study (see Supplemental Figure 8). Therefore, there was no physiological evidence of increased sympathetic activity, or any effect on conduit artery tone, as a result of hypoxia *per se*.

In contrast to radial artery responses, but in keeping with previous studies, the change in FBF in the intervention arm (assessed in the last 3 subjects) was 2.2 ml/min/100 ml greater under hypoxia than normoxia (95% CI 0.6 to 3.8) at the highest dose of nitrite (26 μmol/min) as shown in Supplemental Figure 9. There was no significant difference in baseline FBF between...
hypoxia and normoxia, again showing no physiological evidence of increased sympathetic activity. The overall hemoglobin O₂ saturation recorded during the hypoxia study was 91.6±3.4%, compared to 98.4±0.8% and 100±0.0% in normoxia and hyperoxia, respectively (mean ±SD). As shown in the Supplemental Figure 10, analysis of the ABGs taken at the end of the study, reveal that the pCO₂ was greater during normoxia (5.1±0.2 kPa) than during hypoxia (4.5±0.3 kPa), P=0.015 with no difference in bicarbonate; pH was lower during normoxia (pH 7.396±0.006) compared to pH 7.44±0.01 during hypoxia (P<0.05). Besides pO₂, there were no differences in pH, pCO₂ or plasma bicarbonate under hyperoxia compared to normoxia and hypoxia.

**Study 6 - Fixed dose intra-venous sodium nitrite**

Sodium nitrite was also effective systemically, following intravenous infusion of sodium nitrite (8.7 μmol/min for 60 min) into the contralateral forearm, dilating the radial artery by 10.7% (95% CI 6.8 to 14.7) at 45 min, see Figure 8A.

The profile of the increase in systemic plasma nitrite concentration with intravenous nitrite was similar to the intra-arterial study (see Supplemental Figure 11), although the baseline concentration was higher (0.379±0.065 μmol/L, compared to 0.064±0.019 μmol/L previously) a consequence of the different method (deproteination of plasma using filters versus whole plasma, respectively) the former being typically associated with higher levels in this range, which may be due to contamination of the filters with nitrite. As with the intra-arterial administration of sodium nitrite, intravenous sodium nitrite did not result in any significant changes in peripheral brachial BP (SBP, MAP, or DBP) during the 60 min infusion of sodium nitrite compared to baseline, time 0 min (see Figure 8B). Assessments of central hemodynamics were performed from the third subject onwards (n=7). Sodium nitrite produced large reductions
in central SBP of 11.6 mmHg, (95% CI 2.4 to 20.7), see Figure 8C and peripheral augmentation index (AIx) by 11.9% (95% CI 0.5 to 23.2), see Figure 8D. Nitrite also reduced central pulse pressure (cPP) by 11 mmHg from 28 (19, 38) to 17 (15, 31) median, (25th, 75th percentiles) (P=0.042) and pulse wave velocity (brachial-femoral) by 1.23 m/s (95% CI 0.28 to 2.19).

**Discussion**

These studies have led to several discoveries: (i) inorganic nitrite induces marked dilation of the radial artery (within 2-5 minutes) under normal oxygenated conditions at supra-physiological and near-physiological concentrations (following intra-arterial sodium nitrite administration), (ii) when compared with other vasodilators, nitrite’s actions are highly selective for conduit arteries - to a similar degree as GTN, one of the most selective large/conduit artery dilators identified to date, (iii) nitrite enhanced cGMP production in a dose-dependent manner, suggesting NO-mediated vasodilatory effects, (iv) the effects of nitrite in the radial artery were enhanced by (a) acetazolamide, suggesting a role for carbonic anhydrase, and (b) raloxifene, possibly via inhibition of AO-mediated ROS generation, (v) nitrite-induced radial artery dilation was maximal under conditions of normoxia, being inhibited by hypoxia and hyperoxia, (vi) intravenous sodium nitrite was also an effective systemic conduit artery dilator, causing significant dilation of the contralateral radial artery (~11%), associated with a lowering of cSBP (by ~12 mmHg) and cPP (by ~11 mmHg), at a dose that was not associated with any change in peripheral BP.

The effect of nitrite in the radial artery within minutes, suggests a direct local effect. This was not related to a flow-mediated dilatory mechanism, as no change in conduit artery flow was detected. In addition, the variable selectivity of different vasodilators for conduit versus
resistance vessels indicates that conduit artery dilation occurs independently of forearm blood flow.\(^\text{10}\) For example, alpha blockers and calcium channel blockers are effective at increasing FBF but have minimal effect on conduit artery diameter.\(^\text{10}\) Furthermore, intravenous nitrite (8.7 \(\mu\)mol/min) did not increase FBF systemically, but resulted in radial artery dilation of \(\sim11\%\). Also, intra-arterial co-infusion of acetazolamide enhanced nitrite-induced radial artery dilation, despite causing a borderline reduction in FBF (\(P=0.10\)).

Rather than being a key difference between organic and inorganic nitrates/nitrites as previously thought,\(^\text{23}\) nitrite displayed similar selectivity as GTN, one of the most selective large artery dilators known.\(^\text{10}\) Indeed, it appears that NO donors such as organic nitrates, and to a lesser extent sodium nitroprusside, show greater selectivity for muscular arteries over resistance arterioles, when compared to other vasodilators.\(^\text{10, 13, 24}\) The principle mechanism underlying the vascular effects of organic nitrates is via activation of soluble guanylyl cyclase (sGC), increasing cGMP levels and activating cGMP-dependent protein kinases, and/or cyclic nucleotide-gated ion channels.\(^\text{25, 26}\) These are the same targets for nitrite-derived NO activity. Indeed, intra-arterial nitrite infusion increased local plasma cGMP production in a dose-dependent manner, supporting nitrite-derived NO as the mechanism of dilation. The precise mechanism for nitrite conversion to NO is not clear as a number of enzymes have been implicated in the catalysis of nitrite to NO in different tissue compartments. However, a key pathway considered to account for the effects of nitrite in small resistance vessels is nitrite reduction to NO via deoxyhemoglobin.\(^\text{2}\) In addition, it is possible that nitrite reduction to vasodilating NO is supported by fully oxygenated red cells which possess appreciable nitrite reductase activity in vitro; i.e., \(\sim50\%\) of the capacity of deoxygenated red cells to reduce nitrite (10 \(\mu\)M) to NO,\(^\text{27}\) reflecting the greater reductive potential of heme in the R (oxy) state tetramer.\(^\text{2}\) Thus, HbO\(_2\) saturations \(\sim99\%\) during normoxia.
could support nitrite reduction, although this is difficult to confirm or refute in our in vivo experiments.

However, certain alternative enzymes which may enhance nitrite-derived NO production, or activity, are amenable to pharmacological manipulation in vivo. For example, carbonic anhydrase, an enzyme found in abundance in vessels and RBCs, has been demonstrated by Aamand et al.,18 to possess nitrite anhydrase activity (as the isolated enzyme and in tissue homogenates) increasing NO production, and enhancing nitrite-dependent dilation in the rat aorta (at 1% O2). The CA inhibitor acetazolamide, prevents the hydration of CO2 but not the anhydration of nitrite. It has been suggested that the two substrates bind to different groups in the active site and that acetazolamide may increase the affinity for nitrite, by occupying nonproductive binding sites on the enzyme, thus enhancing its activity as a nitrite anhydrase.18 Notably, such activity appears to predominate around physiological pH, as the initial rates of NO production from nitrite via CA in the presence of acetazolamide were greater at pH 7.2 than pH 5.9, albeit under strict anaerobic conditions. In our studies, administering acetazolamide (either systemically via the oral route, or locally via the intra-arterial route) significantly enhanced radial artery dilation to nitrite, supporting this mechanism of enhanced NO production.

Mechanisms which may enhance nitrite-derived NO activity include inhibition of ROS production. While XO is established as an important source of ROS, the activity of AO to generate ROS is ~25-fold greater than XO in human liver and rat hearts.14 Raloxifene, a potent inhibitor of AO,19 enhanced nitrite-induced dilation of the human radial artery, and the rat aorta suggesting that AO-mediated ROS production predominates over AO-mediated nitrite reduction in normoxia. On the other hand, conditions which increase ROS production such as hyperoxia,28 will diminish nitrite-derived NO activity. Indeed, hyperoxia inhibited nitrite-induced radial artery
dilation.

Hypoxia has also been shown to amplify ROS production.\textsuperscript{29} We therefore propose that this increased ROS production accounts for the attenuated dilatory effect of nitrite in the radial artery - to a similar degree as during hyperoxia, and this overrides the increased rate of nitrite reduction resulting from the greater proportion of deoxyHb encountered under hypoxic conditions in the conduit artery. By contrast, in the resistance vessels, hypoxia augmented nitrite-increased FBF, as reported previously.\textsuperscript{3} This suggests less scavenging of NO in the arterioles, which may be, at least in part, a consequence of the RBC-free zone.\textsuperscript{30} In smaller vessels, a narrower RBC-free zone results in a closer proximity of the RBC to the vessel wall, minimizing the diffusion distance for NO and the duration in which it may be scavenged by ROS. In the larger radial artery, the wider RBC-free zone results in the opposite. Thus, in the resistance arteriole, the effects of enhanced reduction of nitrite via deoxyHb predominates, whereas in the conduit vessel, scavenging by ROS prevails. A further mechanism for nitrite’s selective vascular effects in normoxia versus hypoxia, may involve the recently described endothelial Hbα, which is absent from larger arteries, but present in the myoendothelial junctions of small arteries, where it dampens responses to (eNOS-derived) NO.\textsuperscript{31} However, in hypoxia, it is possible that deoxyHbα supports further nitrite reduction to NO which is in the immediate vicinity of the vascular smooth muscle cells, enhancing vasodilation of resistance arterioles.

Nitrite was effective systemically, with a modest dose of nitrite (8.7 μmol/ min, 0.6 mg/min) selectively dilating the radial artery by \textasciitilde11\% after 45 min with no effect on FBF. The delayed response represents the time for the systemic concentration of nitrite to accumulate, given an initial half-life of nitrite of \textasciitilde20 min (with continuous infusion).\textsuperscript{32} Consistent with this selective dilation of conduit arteries, nitrite selectively lowered cSBP/cPP by \textasciitilde12 mmHg/\textasciitilde11
mmHg respectively with no effect on peripheral BP. Nitrite also reduced peripheral AIx by 11.9±4.6%, which equates to a change in central AIx of ~10%.33 The systolic BP in the proximal aorta (central SBP) differs from brachial systolic BP due to propagation and amplification of pulse pressure from central to peripheral sites. Organic nitrates are known to have a relatively selective effect on central rather than peripheral blood pressure,24,34 thought to be mediated by a dilation of conduit arteries and reduction in pressure waves reflected from conduit arteries back towards the proximal aorta.13 It is therefore likely that the effects of nitrite on central hemodynamics are mediated by a similar mechanism. Central haemodynamics more closely reflect the load on the heart and brain and are at least, if not more than predictive of cardiovascular events than peripheral BP.35 It is surprising that no effect on peripheral BP was seen despite systemic nitrite concentrations ~2.5 μmol/L, which are ~50% greater than those achieved with nitrate supplementation as a source of nitrite (~1.7 μmol/L).20 The lack of effect of nitrite on peripheral BP is consistent with the findings from a recent study performed in 55 patients with peripheral arterial disease, which found no reduction in BP with sustained release sodium nitrite up to doses of 160 mg twice daily.36 The reason for this differential effect presents an enigma and may indeed suggest a more complex interplay between the two anions and other metabolites of the nitrate-nitrite-NO pathway.

Normalization of elevated aortic pulse wave velocity (aPWV, a marker of large elastic artery stiffness) was recently demonstrated in old mice given oral dietary nitrite supplementation for 3-weeks.37 While acutely, dietary nitrate has recently been demonstrated to reduce aPWV by 0.3 m/s in healthy volunteers and by ~0.5 m/s in grade 1 hypertensives, this was in parallel with reductions in brachial systolic pressures of ~5 mmHg and ~12 mmHg respectively.38,39 Our current studies show an acute effect of nitrite, lowering bpmPWV by ~1.2 m/s, independent of
peripheral BP.

Several limitations of this study merit consideration. All studies involved healthy male volunteers. It remains to be determined whether these mechanisms would be similar in patients with vascular disease and hypertension, with particular emphasis on the role of XO, which is upregulated in these groups. Other enzymatic pathways were not tested here (ALDH-2, CYP-450, interaction with PDE5). Also, the contribution of the selective estrogen receptor modulator (SERM) function of raloxifene, relative to its inhibition of AO-induced ROS production, could not be determined; studies in females may help to determine this, as might the specific inhibition of ROS activity. However, the latter is difficult to demonstrate in humans in vivo, as the commonly-used antioxidant, ascorbic acid, is itself a reducing agent which will reduce nitrite to NO and enhance nitrite-induced vasodilation, as previously shown with FBF.32 Whilst we performed a broad range of studies, the sample sizes of most of these were very small and this may affect the validity of the statistical tests. However, many of the p values are substantially below 0.05, which mitigates this concern somewhat.

Conclusions

We have discovered that, contrary to expectation, inorganic nitrite (at supra-physiological and near-physiological concentrations) selectively dilates conduit arteries under normal oxygenated conditions, to a similar degree as GTN, and selectively lowers central systolic/pulse pressure, with important therapeutic potential. Mechanisms associated with nitrite’s activity—include cGMP production and involvement of carbonic anhydrase. In addition, the inhibition of conduit artery dilation in hypoxia and hyperoxia, and the enhancement of dilation by aldehyde oxidase inhibition, suggest an important role for ROS production in modulating these responses.
Acknowledgments: We thank the volunteers for participating in these studies and Mr Paul Seed, Senior Lecturer in Medical Statistics, for expert statistical advice.

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Conflict of Interest Disclosures: King’s College London and PJ Chowienczyk have an interest in Centron Diagnostics Ltd, A King’s College London spin-out exploiting technology for measuring central blood pressure (not used in this study).

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Figure Legends:

Figure 1. Effect of 60 min intrabrachial infusion of sodium nitrite (8.7 μmol/min) on (A) change in radial artery diameter (%), (B) change in forearm blood flow (FBF). Data shown as median and interquartile range in (A), and mean±SEM in (B), n=8, †††P<0.0001 on ANOVA; **P<0.01, ***P<0.001 compared to baseline; #P<0.05, ##P<0.01 compared to 5 min.

Figure 2. Effects of low doses of intrabrachial sodium nitrite on (A) radial artery diameter, nitrite (0.087 μmol/min) (B) forearm blood flow (FBF), nitrite (0.087 μmol/min), (C) RA diameter, nitrite (0.87 μmol/min), and (D) FBF, nitrite (0.87 μmol/min); n=8.

Figure 3. Changes in radial artery diameter (%) with intrabrachial infusions of (A) sodium nitrite and (B) GTN and (C) sodium nitrite and GTN versus change in forearm blood flow (FBF) at each dose step of nitrite (0.087-87 μmol/min) and GTN (0.013-4.4 nmol/min). Data shown as mean±SEM, n=8 (nitrite), n=7 (GTN), **P<0.01, ***P<0.001, compared to baseline, †††P<0.0001 across the dose range.

Figure 4. Effect of intrabrachial infusions of sodium nitrite on (A) plasma nitrite concentration in infused and contralateral arms (n=8), and (B) plasma cGMP production: (i) nitrite (0, 0.26 and 2.6 μmol/min), cGMP in intervention arm (n=6), (ii) nitrite (0, 26 μmol/min), cGMP in intervention arm (n=7), (iii) nitrite (87 μmol/min), cGMP in intervention and control arms (n=5). Data shown as mean±SEM, #P<0.01, ###P<0.001 compared to control arm, **P<0.01, ***P<0.001 compared to nitrite (0 μmol/min), ‡‡P<0.01 for dose response, †††P<0.0001 compared to control arm on ANOVA.
Figure 5. Effect of the administration of (A) oral acetazolamide and (B) oral raloxifene on the change in radial artery diameter (%) during an intrabrachial infusion of sodium nitrite (dose response 0.087-26 μmol/ min). Data shown as mean±SEM, n=14, †P<0.025, †††P<0.001, compared to placebo.

Figure 6. Effect of the intrabrachial administration of acetazolamide (0.1-3 mg/min), nitrite (2.6 μmol/ min) or both in combination on (A) the change in radial artery diameter and (B) the change in forearm blood flow (FBF). Data shown as mean±SEM, n=8, †††P<0.001, *P<0.05 compared to nitrite alone (2-way ANOVA, with Bonferroni post-testing respectively); for acetazolamide alone: ‡P<0.05, #P<0.05 compared to baseline (1-way ANOVA, with Dunnett’s post-testing respectively); (C) change in radial artery diameter over the first 8 minutes of intrabrachial nitrite infusion (2.6 μmol/ min). Data represents mean, recordings every 3 seconds, n=8.

Figure 7. Effect of systemic hypoxia, normoxia and hyperoxia on the change in radial artery diameter during an intrabrachial infusion of sodium nitrite (0.087-26 μmol/ min); n=8, **P<0.01 compared to hypoxia, †††P<0.001 compared to hypoxia and hyperoxia.

Figure 8. Effect of intravenous sodium nitrite (8.7 μmol/ min over 60 min) on (A) change in radial artery diameter (%) in the contralateral arm, (B) peripheral brachial blood pressure (BP) measurements (systolic, SBP, mean arterial, MAP, or diastolic, DBP), (C) central Systolic Blood Pressure (cSBP), and (D) peripheral augmentation index (pAIx) performed before and after the 60 min infusion of sodium nitrite. Data shown as mean±SEM, n=9 A-B, n=7 C-D, *P<0.05, **P<0.01 compared to baseline, †††P<0.001 overall.
Figure 1

(A) Radial artery diameter (%)

(B) Infused arm vs. Control arm

FBF (ml/min/100 ml)
Figure 2
Figure 3
Figure 4
**Figure 5**

**A**

- Acetazolamide
- Placebo

**B**

- Raloxifene
- Placebo
Figure 6
Figure 7
Figure 8
Paradoxical Normoxia-Dependent Selective Actions of Inorganic Nitrite in Human Muscular Conduit Arteries, and Related Selective Actions on Central Blood Pressures
Sami A. Omar, Henry Fok, Katharina D. Tilgner, Ashok Nair, Joanne Hunt, Benyu Jiang, Paul Taylor, Philip Chowienczyk and Andrew Webb

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Supplemental Material
Supplemental Methods

Study Protocols

Participants were asked to avoid ingesting nitrate-rich foods (including green leafy vegetables, beetroot and processed meats), caffeine, smoking and undertaking heavy exercise for 24 hours prior to the study. They were also instructed to fast overnight (though encouraged to take clear fluids to avoid dehydration). The studies were conducted at a similar time of the day for repeated visits in the same subject, under controlled temperatures (23-27 °C) and with minimal sensory stimulation to minimise confounding. For intra-arterial (i.a.) studies, the brachial artery was cannulated using a 27 gauge needle (Cooper’s Needle Works, Birmingham, UK). This part of the procedure was performed with the use of local anaesthesia and under strict aseptic techniques. For intravenous (i.v.) studies, an 18 gauge cannula in an antecubital fossa vein was used. On successful cannulation the needle was secured in position and a saline infusion was commenced at a rate of 1ml/min (Injectomat Agilia syringe driver, Fresenius Kabi, Homburg, GR). Following an initial equilibration period of 15 min and recording of baseline measurements with 0.9% saline, the effects of i.a./i.v. sodium nitrite (Martindale Pharmaceuticals, UK and Ipswich Hospital Pharmacy Manufacturing Unit, UK)/GTN (Hospira Ltd, UK) on radial artery diameter, FBF and systemic hemodynamic parameters were assessed in the studies described in the main paper.

Systemic haemodynamic measurements

Following a minimum period of 5 min laying supine, baseline readings of non-invasive measurements of the following systemic haemodynamics were performed: pulse wave velocity (PWV): brachial-femoral (bfPWV) was measured over the brachial to radial path from simultaneous pressure cuff recordings at each site using the Vicorder system (Skidmore Medical Ltd, Bristol UK). The path distance was taken from the proximal edge of the upper arm cuff to that of the wrist cuff. Peripheral blood pressure and heart rate were also measured (IntelliVue MP30, Phillips, NL): during forearm blood flow studies, a thigh cuff
was placed over the arm cuff, however, measurements were also taken at baseline and at the end of the infusion with the BP cuff placed directly on the arm; these latter measurements were used to calibrate the Finometer® (Finapres Medical systems, Amsterdam NL) used to determine central blood pressure, augmentation index and heart rate. An average of two readings was taken for the final results, due to time limitations. Measurements were repeated during and/or at the end of the study. In calculating the cPP, the peripheral DBP measurement was used, as central DBP was not recorded; however, DBP changes by only 1-2 mmHg from the aorta to the periphery, and therefore our measurement of peripheral DBP is a close estimate of central DBP. In addition, SpO2, SpMet (Radical-7, Rainbow, CA, US) were measured continuously in Studies 1, 2, 5 and 6.

**Measurement of local Radial Artery Diameter (conduit vessel)**

The radial artery was imaged using an Acuson-Aspen advanced 2-dimensional ultrasound with 10 MHz Linear probe (Siemens GR), which was fixed in position using a magnetic flexible stand (Mitutoyo JP). The image was acquired at the end-diastolic phase (ECG triggered) for 120 s at a rate of 1 frame every 3 s giving a total of 40 frames per sequence of acquisition. The Radial artery diameter was measured using computer aids edge detecting software (Brachial analyzer, Medical Imaging Applications) an average of those measurements was used to give the vessel diameter at that time point.

**Measurement of local Forearm Blood Flow (resistance arterioles)**

Forearm blood flow (resistance arterioles) was measured using venous occlusion plethysmography as we have described previously. The volunteer remained supine. Blood pressure cuffs were placed around the upper arms and the wrists. The cuffs themselves were connected to an E20 Rapid Cuff Inflators which in turn were supplied by an AG101 Cuff Inflator Air Source (Hokanson Inc. WA US). The circumference of the forearms was measured using a mercury-in-silastic strain gauge placed around each forearm connected to an EC6 Plethysmograph (Hokanson Inc. WA US). To measure forearm blood flow (FBF) the wrist cuffs were inflated to supra-systolic pressure (180±2 mmHg) to exclude the circulation
in the hand. After a 30-60 s pause to allow the changes in the forearm circulation to reach equilibrium, the cuffs around the upper arm were inflated to 40±2 mmHg) and deflated a number of times in cycles consisting of ~10 s inflation and ~5 s deflation. The degree of change in the circumferential size of the forearm measured by the mercury-in-silastic strain gauge was acquired by channelling the signal through the EC-6 Plethysmograph, and recorded and analysed using Chart 5 software to give a value of FBF in ml of blood / 100ml tissue / s.

In the dose-response studies, radial artery responses were assessed at 5-7 min following each change in dose, while FBF changes were assessed at 20-22 min following each change in dose unless otherwise stated, with the change in the next dose occurring at 25 min.

Venous capacitance was measured by keeping the arm cuffs inflated at 40 mmHg and assessing changes in forearm volume at 2.5 minutes as previously described. During i.a. infusion of the low dose series of nitrite (n=3) and i.a. infusion of GTN (n=3).

**Hypoxia/Normoxia/Hyperoxia studies**

A CPAP mask fixed with a one way 5.0 cm H₂O PEEP valve system was securely fitted around the participant's nose and mouth. The mask was connected via 1.5 meters of tubing to either: a room air port (normoxia), a 100% oxygen port (hyperoxia) or a 12% oxygen/nitrogen balanced gas cylinder (hypoxia), the latter to achieve stable arterial oxygen saturation levels (as measured by pulse oximetry) above 83%, similar to that described previously. All three inhaled gases were delivered at a pressure of 1.5 to 2 bars and the flow of gas was adjusted to achieve the desired oxygen saturations at a level that was comfortable for the volunteer (15 – 20 liters/minute). At the completion of the study an arterial blood gas sample was aspirated via the brachial needle and analysed through a blood gas machine.
**Measurement of plasma nitrite**

Blood was sampled serially through an 18 gauge Venflon® cannula placed in a forearm vein, in some studies blood was taken from the arm receiving the infusion (ipsilateral arm) in addition to the contralateral arm. Five ml samples of blood were aspirated and immediately transferred to pre-chilled Lithium Heparin tubes (Vacutette) and immediately spun at 4 °C for 5 min at 4700 RPM (Mikro 220R centrifuge, Hettich GR). The haemolysis-free supernatant plasma was removed and placed into two 1.5 ml pre-labelled Ependorff tubes. These samples were snap-frozen in liquid nitrogen and stored at -80°C until the day of the analysis, when they were thawed and stored on ice. The levels of nitrite in whole plasma were analysed using the 280i Nitric Oxide Analyzer (NOA) (Sievers Instruments, GE analytic instruments). The Purge vessel was filled with sodium iodide dissolved in 99.8% glacial acetic acid to which a few drops of antifoaming agent were added. To generate a calibration curve, a stock solution of 100 mM sodium nitrite was used to prepare the standards which were used in generating the calibration curves. The series of standard dilutions was constructed by serial dilution of the stock solution giving concentrations of 100 nM, 0.5 µM, 1 µM, 5 µM, 10 µM, 50 µM, 100 µM. HPLC nitrite free water was used in the preparation of the stock and standard solutions. Using an analytical syringe, 50 µL samples of selected dilutions were injected, in duplicate, into the purge vessel directly onto the reducing agent and the NO generated was detected by the NOA and analysed by the software to generate the calibration curve. To maintain consistency, close attention was paid to the volume of reducing agent, the maintenance of the cell pressure and the maintenance of the gas pressure. Once a satisfactory calibration curve had been generated, 50 µL of the plasma samples were injected, in duplicate, into the purge vessel. The reducing agent in the purge vessel was changed after each duplicate. The concentrations of nitrite were derived from the calibration curve. For most studies whole plasma was used, with antifoam added to the chamber to reduce frothing. In study 6, plasma was deproteinated using 3K Microcon filters (Merck Millipore).
**Measurement of Plasma SNO’s**

Using an 18 gauge needle, blood samples were collected from the contralateral arm at baseline and from the infused arm at the end of the study. Blood (5 ml) samples were aspirated and treated in an identical manner as those described above for plasma nitrite. On the day of the analysis the samples were thawed and placed on ice. At least three minutes prior to analysis part of the sample was treated with 5% acidified sulfanilamide (in a ratio of 9:1). Samples were analysed using the 280i Nitric Oxide Analyzer (NOA) (Sievers Instruments, GE analytic instruments) as per nitrite. However, in this case the Purge vessel was filled with potassium tri-iodide dissolved in distilled water and 99.8% glacial acetic acid to which a few drops of antifoaming agent were added. The remainder of the analysis was conducted in a manner identical to the one described above for plasma nitrite.

**Measurement of cGMP levels**

Blood samples (~4 ml each) were collected at appropriate time points, via an 18 gauge cannula, placed in the anticubital fossa vein, of either the infused or contralateral arm and transferred to pre-chilled EDTA tubes (Vacuette) and immediately spun at 4 °C for 5 min at 4700 RPM (Mikro 220R centrifuge, Hettich GR). The haemolysis-free supernatant plasma was removed and placed into two 1.5 ml pre-labelled Ependorff tubes. These samples were snap-frozen in liquid nitrogen and stored at -80°C until the day of the analysis. The assessment was conducted using the Amersham cGMP Enzymeimmunoassay Biotrak system (GE Healthcare). The reagents provided, in the assay kit, were allowed to equilibrate to room temperature prior to use. Diluted assay buffer was then reconstituted by adding - and thoroughly mixing, the contents of the provided concentrate to distilled water, bringing the total volume to 500 ml. The dilute wash buffer was reconstituted in an identical manner. Both the lyophilised cGMP antibody and the lyophilised cGMP conjugate were reconstituted by adding 11 ml of the diluted assay buffer to each of the individual bottles and gently mixed until completely dissolved, taking care to avoid excessive agitation and foaming. The stock standard of cGMP (10.24 pmol/ml) was reconstituted by adding 2.5 ml of the diluted assay
buffer to the acetylation standard bottle; the contents were carefully mixed until completely dissolved. Eight further working standards were constructed by consecutive serial dilutions of the stock solution, with an equal volume of dilute assay buffer, giving concentrations of 5.12, 2.56, 1.28, 0.64, 0.32, 0.16, 0.08 and 0.04 pmol/ml. The plasma samples for analysis (or unknowns) were prepared by diluting the plasma with the assay buffer in a ratio of 10:1. A volume of 1 ml of each of the standards and each of the unknowns was transferred to a polypropylene tube and labelled appropriately. The acetylation reagent was then constituted by adding 2.5 ml of acetic anhydride to 5 ml of triethylamine in a glass vessel and mixing thoroughly. The resulting reagent was added in aliquots (100 μL) to each of the polypropylene tubes, containing 1 ml volumes of the standards or unknowns; each tube was then immediately agitated using a vortex mixer. The pre-treated micro-plate was set up with sufficient wells to run a non-specific binding (NSB) blanks, standards and unknowns. Excluding the wells designated as NSB, 100 μL of the diluted antiserum was added to all the wells. This was followed by adding 150 μL of the assay buffer to the NSB wells and 50 μL aliquots from all the acetylated standards and diluted plasma samples to the appropriate pre-designated wells. All samples were added in duplicate. On completion the microplate was covered, gently mixed and incubated at 3 – 5 °C for 2 hours. Aliquots of the dilute conjugate (100 μL) were then added to each of the wells, and the micro-plate was covered, gently mixed and allowed to incubate at 3 – 5 °C for 1 hour. At the completion of this time period the plate was carefully and thoroughly washed using the diluted wash buffer. The plate was blotted to ensure the removal of any residual wash buffer. Room temperature tetramethylbenzidine (TMB) hydrogen peroxide was immediately added to the wells in 200 μL aliquots. The micro-plate was covered and agitated at room temperature on a shaker for 30 min. The reaction was halted by adding 100 μL of 1M sulphuric acid to all the wells. The end point value was determined using a spectrophotometric plate reader capable of measuring optical density at 450 nm. SoftMax Pro ® microplate acquisition and analysis software was used to interpret the data.
Study 2(aji) – pH Buffered Saline

Given that 0.9% saline has a pH of ~5.5 (though is rapidly buffered in the circulation in vivo), and could therefore result in acidic disproportionation of nitrite, in a separate series of experiments we pH-balanced the saline by adding small amounts of sodium bicarbonate to achieve a pH ~7.5 and tested the effect on nitrite-induced radial artery dilatation as before.

Study 4 – continuous imaging of radial artery

As near maximal dilation was achieved at 5 minutes in previous studies here we elected to capture a continuous US image of the radial artery during the first 8 minutes after the commencement of the fixed dose (2.6 µmol/ml) i.a. nitrite infusion.

Rat Thoracic Aorta Myography Protocol

In vitro large artery function experiments were performed employing a Model 700MO Tissue Organ Bath System (Danish Nyo Technology, Aarhus DK) on aortic rings obtained from Sprague Dawley rats. The animals were killed by exposure to raising concentration of CO₂; the heart and aorta were removed and placed in ice cold Physiological Salt Solution (PSS). The thoracic aorta was cleaned of fat and adherent tissue while immersed in cold PSS buffer. Four aortic rings measuring 2.5 mm in length were cut using a scalpel and rings were then individually mounted in separate organ baths, containing 5 ml of PSS bubbled with 95% O₂/5% CO₂ (pH 7.3-7.4) maintained at a temperature of 37 °C (Organ Bath Model 700 MO, Danish Nyo Technology, Aarhus, Denmark, incorporating Myodaq 2.01 acquisition and Myodata 2.02 analysis software).

Vessels were slowly stretched to 5 mN in a stepwise manner of 1mN increments every 5 minutes. Vessels were allowed to equilibrate at 5mN over 30 min. Care was taken to change the organ bath’s PSS buffer every 15 minutes throughout this procedure.

Following equilibration the vessels were put through a standard run up procedure in which they were maximally constricted for a duration of 3 minutes by 3 consecutive 125 mM KPSS
challenges 10 minutes apart and separated by repeated PSS washouts a minimum of 3 times and until baseline tension was reached.

Following the standard run up procedure, dose response curves to phenylephrine ($10^{-9} - 10^{-5}$ M) were constructed for each ring. Phenylephrine was added to the organ baths in cumulative half log molar doses every 3 minutes followed by wash-out.

Using the constructed dose vs tension curve the dose of phenylephrine required to achieve 80% of the maximum response (80% $\text{PE}_{\text{max}}$) was established.

Either acetazolamide (final concentration: $10^{-7}$, $10^{-6.5}$ and $10^{-6}$ M) dissolved in PSS, or raloxifene (final concentration: $10^{-7}$, $10^{-6.5}$ and $10^{-6}$ M), initially dissolved in DMSO, was added to three of the four organ baths, the remaining organ bath contained vehicle control.

Aortic rings were submaximally pre-contracted to 80% $\text{PE}_{\text{max}}$ for 3 minutes and then dose response curves were constructed to sodium nitrite ($10^{-9} - 10^{-5}$ M) in cumulative half log molar doses.

The effects of Acetazolamide on vessel relaxation to NaNO$_2$ were compared using multiple regression with dummy variables for the different concentrations of NaNO$_2$ (9 levels, up to $10^{-3}$ M) and ACZ (4 concentrations), and for differences between animals (5 animals). This permitted a small number of strong tests using all the available information, rather than a large number of weaker tests requiring adjustment for multiple comparisons. No correction was made for clustering by animal, due to the fully balanced design.
Supplemental Results

Supplemental Table 1.

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<th>Study 2a i.a. nitrite</th>
<th>Study 2ai i.a. nitrite</th>
<th>Study 2b i.a. GTN</th>
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Supplemental Table 1. Demographic data. Data shown as mean±SD. NR = not recorded, i.a. = intra-arterial, i.v. = intravenous, ACZ = acetazolamide. O₂ = Oxygen.
Supplemental Figure Legends

Supplemental Figure 1. Effect of 60 min intrabrachial infusion of sodium nitrite (8.7 µmol/min) on peripheral brachial blood pressure (systolic, SBP, mean arterial, MAP, or diastolic, DBP). Data shown as mean±SEM, n=7 in (A) n=8 in (B).

Supplemental Figure 2. Effect of intrabrachial infusion of sodium nitrite (dose response 0.087-26 µmol/min) in pH-buffered saline on change in radial artery (RA) diameter (%). Data shown as mean±SEM, n=4, *P<0.05, **P<0.01, ***P<0.001, compared to baseline.

Supplemental Figure 3. Effect on Forearm blood flow (FBF) of intrabrachial infusion (A) sodium nitrite (0.087-2.6 µmol/min), n=8, and (B) GTN (0.013-4.4 nmol/min), n=7.

Supplemental Figure 4 Effect of intrabrachial infusion of sodium nitrite (0.087-87 µmol/min) on percentage change in radial artery (RA) mean flow.

Supplemental Figure 5. Venous Capacitance: change in forearm volume with intrabrachial infusions of (A) sodium nitrite (0.087-2.6 µmol/min) and (B) GTN (0.013-4.4 nmol/min). Data shown as mean±SEM, n=3 in both groups; †P<0.05, ‡P<0.01 compared to baseline (1-way ANOVA).

Supplemental Figure 6. Effect of intrabrachial infusion of sodium nitrite (dose response 0.087-87 µmol/min) on percentage methemoglobin (MetHb (%)) in infused and control arm (left Y-axis) and percentage hemoglobin oxygen saturations (SaO2 (%)) in control arm (right Y-axis). Data shown as mean±SEM, n=8, *P<0.05, ***P<0.001, compared to baseline.
Supplemental Figure 7. Mechanisms of nitrite-induced dilation of phenylephrine (PE)-constricted rat aorta: (a) acetazolamide (ACZ, $10^{-6} – 10^{-7}$ mol/L), (b) raloxifene (RAL, $10^{-6} – 10^{-7}$ mol/L). Data shown as mean±SEM, n=5, †††P<0.001 compared to control on ANOVA.

Supplemental Figure 8. Effect of hypoxia v normoxia v hyperoxia during an intrabrachial infusion of sodium nitrite (0.087-26 µmol/ min) on peripheral brachial blood pressure, BP, ((A) systolic, SBP, (B) diastolic, DBP, (C) mean arterial, MABP and (D) heart rate (HR). Data shown as mean±SEM, n=8, **P<0.01 compared to pre-nitrite (1-way ANOVA, with Bonferroni multiple post-testing).

Supplemental Figure 9. Effect of systemic hypoxia and normoxia on the change in forearm blood flow during an intrabrachial infusion of sodium nitrite (0.087-26 µmol/ min). Data shown as mean±SEM, n=3, **P<0.01 compared to normoxia.

Supplemental Figure 10. Effect of hypoxia v normoxia v hyperoxia on arterial blood gas parameters: (A) $O_2$ partial pressure, (B) $CO_2$ partial pressure, (C) pH, (D) $HCO_3^-$.

Supplemental Figure 11. Effect of intravenous sodium nitrite (8.7 µmol/ min over 60 min) on systemic plasma nitrite concentrations (blood sampled from veins in contralateral arm, assay performed using deproteinated plasma). Data shown as mean±SEM, n=7, **P<0.01 compared to baseline
Supplemental Figure 1

![Blood pressure graph showing MAP, SBP, and DBP over time.](image-url)
Supplemental Figure 2

Graph showing the relationship between nitrite concentration (µmol/min) and radial artery diameter (%). The x-axis represents nitrite concentration, and the y-axis represents radial artery diameter. The data points are connected by a line, and error bars indicate variability. Significant differences are indicated by asterisks: * for p < 0.05, ** for p < 0.01, and *** for p < 0.001.
Supplemental Figure 3

A

Nitrite (μmol/min)

Δ FBF (ml/min/100 ml)

0
1
2
3
4
5
6
7

0 0.087 0.26 0.87 2.6 8.7 26 87
***
***
***
**

B

GTN (nmol/min)

Δ FBF (ml/min/100 ml)

0
1
2
3
4
5

0 0.013 0.044 0.13 0.44 1.3 4.4
***
***
***

A

B
Supplemental Figure 5

A

Δ Forearm Volume (ml/100 ml)

Control
Nitrite

Nitrite (µmol/min)

B

Δ Forearm Volume (ml/100 ml)

Control
GTN

GTN (µmol/min)
Supplemental Figure 6

- SaO2 (%) - Control arm
- MetHb (%) - Infused arm
- MetHb (%) - Control arm

Nitrite (µmol/min)

MetHb (%)

SaO2 (%)
Supplemental Figure 7

A

B
Supplemental Figure 9

Nitrite (µmol/min)

δ FBF (ml/min/100 ml)

Hypoxia

Normoxia

Nitrite (µmol/min)
Supplemental Figure 10

A

B

C

D

PO₂ (kPa)

PCO₂ (kPa)

pH

HCO₃⁻ (mmol/L)

Hypoxia

Normoxia

Hyperoxia

7.40

7.45

7.50

7.60

7.65

7.70

7.80

7.85

7.90

20

40

60

4.0

4.5

5.0

5.5

6.0

4.5

5.0

5.5

6.0

20

21

22

23

24

25

26
Supplemental Figure 11

![Graph showing the increase in nitrite concentration over time (in μmol/L) with error bars. The x-axis represents time in minutes (0-60) and the y-axis represents nitrite concentration (0-6). Two asterisks indicate significant differences at certain time points.](image-url)
Reference List

