

Circulation

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Hypoxic Modulation of Exogenous Nitrite-Induced Vasodilation in Humans

Abdul R. Maher, Alexandra B. Milsom, Prasad Gunaruwan, Khalid Abozguia, Ibrar Ahmed, Rebekah A. Weaver, Philip Thomas, Houman Ashrafian, Gustav V.R. Born, Philip E. James and Michael P. Frenneaux

Circulation. 2008;117:670-677; originally published online January 22, 2008;

doi: 10.1161/CIRCULATIONAHA.107.719591

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2008 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circ.ahajournals.org/content/117/5/670>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:

<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Circulation* is online at:

<http://circ.ahajournals.org/subscriptions/>

Hypoxic Modulation of Exogenous Nitrite-Induced Vasodilation in Humans

Abdul R. Maher, MRCP; Alexandra B. Milsom, PhD; Prasad Gunaruwan, MRCP; Khalid Abozguia, MRCP; Ibrar Ahmed, MRCP; Rebekah A. Weaver, MSc; Philip Thomas, BMedSci; Houman Ashrafian, MRCP; Gustav V.R. Born, PhD; Philip E. James, PhD; Michael P. Frenneaux, MD

Background—It has been proposed that under hypoxic conditions, nitrite may release nitric oxide, which causes potent vasodilation. We hypothesized that nitrite would have a greater dilator effect in capacitance than in resistance vessels because of lower oxygen tension and that resistance-vessel dilation should become more pronounced during hypoxemia. The effect of intra-arterial infusion of nitrite on forearm blood flow and forearm venous volumes was assessed during normoxia and hypoxia.

Methods and Results—Forty healthy volunteers were studied. After baseline infusion of 0.9% saline, sodium nitrite was infused at incremental doses from 40 nmol/min to 7.84 $\mu\text{mol}/\text{min}$. At each stage, forearm blood flow was measured by strain-gauge plethysmography. Forearm venous volume was assessed by radionuclide plethysmography. Changes in forearm blood flow and forearm venous volume in the infused arm were corrected for those in the control arm. The peak percentage of venodilation during normoxia was $35.8 \pm 3.4\%$ (mean \pm SEM) at 7.84 $\mu\text{mol}/\text{min}$ ($P < 0.001$) and was similar during hypoxia. In normoxia, arterial blood flow, assessed by the forearm blood flow ratio, increased from 1.04 ± 0.09 (baseline) to 1.62 ± 0.18 (nitrite; $P < 0.05$) versus 1.07 ± 0.09 (baseline) to 2.37 ± 0.15 (nitrite; $P < 0.005$) during hypoxia. This result was recapitulated in vitro in vascular rings.

Conclusions—Nitrite is a potent venodilator in normoxia and hypoxia. Arteries are modestly affected in normoxia but potently dilated in hypoxia, which suggests the important phenomenon of hypoxic augmentation of nitrite-mediated vasodilation in vivo. The use of nitrite as a selective arterial vasodilator in ischemic territories and as a potent venodilator in heart failure has therapeutic implications. (*Circulation*. 2008;117:670-677.)

Key Words: nitric oxide ■ nitrites ■ vasodilation ■ heart failure ■ veins ■ hypoxia

The ability of blood vessels to match oxygen delivery to metabolic demand by vasodilation in response to hypoxia is well described. It has been hypothesized that retrograde propagation of vasodilating agents from capillaries or veins to precapillary resistance vessels ensures optimal flow/demand matching.¹ The nature of the signal remains imprecisely defined, and hypoxic vasodilation tends to persist even after pharmacological blockade of known mediators.² The current consensus is that “multiple overlapping and integrated mechanisms or other undiscovered pathways exist, which subserve hypoxic vasodilation.”³ Nitric oxide (NO) may be an essential mediator of the vasodilatory response to hypoxia that may be formed locally or transported to areas of low oxygen tension, resulting in vasodilation.²

Editorial p 594 Clinical Perspective p 677

Controversy surrounds the relative importance of different endogenous NO species in mediating physiological

vasodilation. These species include nitrite, *S*-nitrosothiols, *N*-nitrosamines, iron nitrosyls, and nitrated lipid.² Nitrite has traditionally been considered a weak vasodilator. In aortic specimens, concentrations of nitrite as high as 100 to 1000 $\mu\text{mol}/\text{L}$ are typically required under normoxic conditions to induce relaxation.^{2,4} The discrepancy between plasma nitrite levels in vivo and the levels required to elicit a biological effect in vitro, coupled with a study that demonstrated a lack of vasodilator activity of 200 $\mu\text{mol}/\text{L}$ nitrite in the forearms of healthy volunteers, diminished interest in nitrite.⁵ In contrast, a greater focus was directed to hemoglobin acting as an NO store, oxygen sensor, and condition-sensitive NO donor, operating through release of the NO group from *S*-nitrosohemoglobin.⁶

More recently, nitrite has been recognized as a powerful signaling molecule and regulator of gene expression.^{7,8} Although in normoxia, nitrite is a relatively inefficacious vasodilator when acting directly, during hypoxia, this action

Received June 12, 2007; accepted November 16, 2007.

From Birmingham University (A.R.M., P.G., K.A., I.A., R.A.W., P.T., M.P.F.), Birmingham, United Kingdom; School of Medicine, Cardiff University (A.B.M., P.E.J.), Cardiff, United Kingdom; Department of Cardiovascular Medicine (H.A.), University of Oxford, Oxford, United Kingdom; and William Harvey Research Institute (G.V.R.B.), London, United Kingdom.

Correspondence to Professor Michael P. Frenneaux, MD, Department of Cardiovascular Medicine, The Medical School, University of Birmingham, Birmingham, B15 2TT, United Kingdom. E-mail m.p.frenneaux@bham.ac.uk

© 2008 American Heart Association, Inc.

Circulation is available at <http://circ.ahajournals.org>

DOI: 10.1161/CIRCULATIONAHA.107.719591

may be enhanced. NO released from nitrite as a result of a hypoxic environment may play an important physiological role.² Arteriovenous gradients of nitrite in the human forearm have been observed; these gradients are enhanced during exercise and during regional NO synthase inhibition.^{9,10} Thus, it has been suggested that nitrite is consumed to liberate bioactive NO.^{2,9,10} A vasoactive role for nitrite at normal physiological concentrations is still disputed, because the arteriovenous gradient in nitrite may result from decreased venous nitrite production through decreased NO synthase activity coupled with increased venous nitrite consumption.¹¹

It has been proposed recently that deoxyhemoglobin is capable of acting as a nitrite reductase; hence, hemoglobin would act as an "oxygen sensor" and would liberate NO from nitrite along the physiological oxygen gradient. The work of Gladwin and others² is at least hypothesis-generating with respect to a role for nitrite in the modulation of vascular tone *in vivo*.

In support of an enhanced role for nitrite in hypoxia, *in vitro* nitrite administration has been shown to confer protection against ischemia/reperfusion injury in the heart, kidney, brain, and liver.^{12–15} In addition, nitrite treatment has also proved beneficial in models of hypertension,¹⁶ pulmonary hypertension,¹⁷ and cerebral vasospasm.¹⁸

Accordingly, we hypothesized the following: (1) While subjects were breathing room air, nitrite would be a potent dilator of capacitance vessels *in vivo* because of their relatively low ambient oxygen tension; (2) when subjects were breathing 12% oxygen, which would render the resistance vessels relatively hypoxic, nitrite would become a potent arteriolar dilator. The present study focused on the forearm vascular bed, the small veins and venules of which are representative of vessels that constitute the bulk of the capacitance bed and which behave quite differently from conduit veins such as dorsal hand veins.¹⁹ To test the direct effect of nitrite on vascular tissue (hemoglobin independent), isolated rabbit vessels (aorta and vena cava) were exposed to a range of nitrite concentrations in both normoxia and hypoxia.

Methods

Subjects

Twenty-six healthy volunteers were recruited to protocol A and 14 to protocol B. Subjects had no history of active smoking, hypertension, diabetes mellitus, or hypercholesterolemia and no family history of ischemic heart disease. None took cardioactive medication or vitamin supplements. All had a normal cardiovascular examination and ECG and gave written informed consent. The study was approved by the local research ethics committee. Investigations were performed at the University of Birmingham Clinical Research Block in a dedicated vascular laboratory (22°C to 24°C), with subjects having had a light breakfast and having abstained from caffeine-containing drinks for at least 6 hours.

Measurement of Venous Volume

Forearm venous volume (FVV) was assessed with radionuclide plethysmography. The details and advantages of the technique are detailed elsewhere.²⁰ Briefly, after the administration of stannous fluoride, venous blood was withdrawn, radiolabeled with technetium-99m, and then reinjected into the subject after 10 minutes' incubation. After 15 minutes for stabilization, images were obtained. A region of interest was defined, and images were obtained during

1-minute acquisition periods. At least 90% of the injected isotope was confined to the intravascular space; therefore, forearm radioactive counts were proportional to forearm blood volume. Because the vast majority of blood in the peripheral circulation is contained within the veins, changes in counts reflect changes in FVV.²⁰ During each cycle of acquisition, cuffs were inflated over both upper arms at pressures of 0, 10, 20, and 30 mm Hg. From these data, scintigraphic vascular volumes were plotted against cuff pressure to form a venous pressure-volume relationship. A parallel shift of the relationship indicated a change in venous tone.²⁰ Data were acquired from both the study and control arms, and any changes observed were corrected for those that occurred in the control arm. We and others have previously validated this technique.^{20–22} Importantly, we have shown that the position of the pressure-volume relationship is not altered by large changes in arterial inflow.¹⁹ To allow presentation of grouped data, results were presented as percentages. The data were corrected for physical decay of technetium.

Biochemistry

Chemicals and Reagents

Chemicals were purchased from Sigma (St Louis, Mo), other than glacial acetic acid, high-performance liquid chromatography-grade nitrite-free water, and hydrochloric acid, which were purchased from Fisher Scientific (Loughborough, United Kingdom). Sodium nitrite in the human studies was purchased from Martindale Pharmaceuticals, UK (Brentwood, United Kingdom).

Ozone-Based Chemiluminescence

Plasma nitrite and protein-bound NO were measured with a triiodide reagent linked to ozone-based chemiluminescence, as described previously.²³ The triiodide reagent is probably the most widely used in the NO metabolite field and has been validated against standards in several laboratories.^{10,24–27} The reader is referred to a complete discussion of the assay.^{6,23,28–33}

Stock solution of triiodide reagent was prepared fresh each day.^{23,27} Immediately before analysis, frozen plasma samples were thawed in a water bath at 37°C for 3 minutes. We have previously shown that sample freezing has no effect on plasma NO metabolite stability.³⁴ The area under the curve was used in analyses, and concentrations were calculated from a standard curve of sodium nitrite.

Study Protocols

Protocol A (Normoxia)

Subjects were rested in a supine position. Both forearms were positioned on the face of a gamma camera (Scinticon, MiE America, Inc, Elk Grove Village, Ill, and ADAC-Transcam, Aalborg, Denmark). A 20-gauge venous cannula was inserted into an antecubital vein in each arm. Blood was drawn and sent for a full blood count and biochemical profile. A 27-gauge arterial needle (Coopers Engineering, Birmingham, United Kingdom) mounted onto a 16-gauge epidural catheter and sealed with dental wax was then inserted into the nondominant brachial artery under sterile conditions and kept patent by the continuous infusion of normal saline.

Heart rate and rhythm were monitored continuously (GE Marquette Dash 3000, GE Healthcare, Chalfont St Giles, United Kingdom). Blood pressure was monitored continuously with finger plethysmography (TNO TPD Biomedical Instrumentation, Delft, Netherlands). Mercury-in-Silastic strain gauges were used for the measurement of forearm blood flow (FBF; DE Hokanson, Inc, Bellevue, Wash), as described previously.³⁵ Changes in FBF observed in the study arm were corrected for those that occurred in the control arm and expressed as a ratio (FBFR; infused arm:control arm). Two venous pressure-volume relationships were recorded, and FBF was assessed during the infusion of normal saline. Blood was drawn from both venous cannulas (infusion and control arm) at baseline and at each stage of the study. Blood samples were transferred after blood gas analysis (Bayer Rapidlab 865, Siemens, Tarrytown, NY) into 4-mL EDTA collection tubes and were centrifuged at 2000 rpm for 10 minutes at 4°C. Plasma was snap-frozen in liquid nitrogen and stored

Table 1. Grouped Baseline Characteristics for All Subjects

Demographic and Clinical Features	
Age, y	56.5±1.4
Sex, M/F, n	27/6
Body mass index, kg/m ²	27.0±0.6
Smoking history, n	
Nonsmoker	32
Previous smoker	1
Current smoker	0
Serum cholesterol, mmol/L	5.6±0.2
Plasma glucose, mmol/L	4.8±0.1
Serum creatinine, μmol/L	99.6±3.1
Heart rate, bpm	64.0±2.0
Blood pressure, mm Hg	
Systolic	129±2.2
Diastolic	73.1±1.7
Left ventricular ejection fraction, %	58.3±2.7

Continuous data are presented as mean±SEM.

at -80°C for subsequent analysis. Plasma nitrite and protein-bound NO were measured at each stage in both protocols.

Intrabradial nitrite infusion was then commenced at a dose of 40 nmol/min for 30 minutes. FVV was assessed at 5, 12, and 20 minutes, followed by assessment of FBF. The above was then repeated with doses of nitrite of 100 nmol/min, 314 nmol/min, 784 nmol/min, 3.14 μmol/min, and 7.84 μmol/min. Left ventricular ejection fraction was calculated by multiple-gated acquisition. In a subset of subjects (n=3), radial arterial samples were taken at baseline and at peak nitrite infusion in which levels of nitrite and protein-bound NO were measured.

Protocol B (Hypoxia)

Subjects were prepared as for protocol A. Oxygen saturation levels were monitored continuously with pulse oximetry (Nellcor N-180, Nellcor, Pleasanton, Calif). Baseline measurements of all parameters were taken during the initial saline infusion.

Hypoxia was induced with the subject breathing 12% oxygen via a facemask connected to a 2-way valve. When arterial oxygen saturation levels (as measured by pulse oximetry) were stable in the 83% to 88% range (estimated $pO_2=47.5$ to 55 mm Hg, based on Severinghaus' equation), FVV and FBF were assessed during saline infusion ("hypoxia alone") before the infusion of nitrite.

In 7 subjects, intrabradial nitrite was infused at a dose of 7.84 μmol/min for 20 minutes in either normoxia or hypoxia, in random order. Saline was infused between the 2 infusions of nitrite for 60 minutes. FVV was assessed at 10 minutes into each nitrite infusion, followed by assessment of FBF. In the remaining 7 subjects, protocol B was performed with nitrite at a dose of 314 nmol/min to investigate the effects of hypoxia in the context of a plasma concentration of nitrite close to that found physiologically.

Vascular Tissue Assay

Male New Zealand White rabbits (weight 2 to 2.5 kg) were terminally anesthetized by intravenous injection of sodium pentobarbital (0.75 mL/kg). The abdominal aorta and vena cava were excised and placed in fresh Krebs buffer (NaCl 109.0 mmol/L, KCl 2.7 mmol/L, KH₂PO₄ 1.2 mmol/L, MgSO₄·7H₂O 1.2 mmol/L, NaHCO₃ 25.0 mmol/L, C₆H₁₂O₆ 11.0 mmol/L, CaCl₂·2H₂O 1.5 mmol/L). Vessels were cleaned and cut into 2-mm rings. Rings were mounted on matched stainless steel hooks for isometric tension recording in 8-mL baths containing 5 mL of Krebs buffer.

Tissue was allowed to equilibrate for 60 minutes under a resting tension of 2g at 37°C, the buffer being exchanged every 15 minutes. After final adjustment of the passive tension to 2g, vascular segments

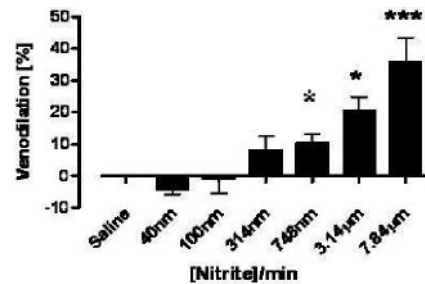


Figure 1. Change in peak FVV under normoxic conditions. Data are expressed as percentage change from baseline (saline infusion). All values are presented as mean±SEM. * $P<0.05$ and *** $P<0.001$ vs saline infusion.

were constricted with phenylephrine (1 μmol/L). Once tension had reached a plateau, acetylcholine (10 μmol/L) was added to demonstrate endothelium viability.

After extensive washout, under normoxic conditions (95% O₂/5% CO₂), vessels were again constricted with 1 μmol/L phenylephrine, and sodium nitrite in Krebs buffer (10, 100, and 1000 μmol/L final concentration) was administered. Under hypoxic conditions (95% N₂/5% CO₂, resulting in ≈1% tissue bath O₂), the tissue was first incubated for 10 minutes before exposure to 3 μmol/L phenylephrine (to achieve a similar gram tension observed at 95% O₂) before administration of sodium nitrite. Tension was recorded for a further 20 minutes with Chart for Windows (ADInstruments, Chalgrove, United Kingdom). The minimum tension achieved was used in all calculations. All relaxations are presented as a percentage of the maximum tension induced by phenylephrine under the relevant conditions.

Data and Statistical Analysis

All data are expressed as mean±SEM, and probability values <0.05 were considered statistically significant. Repeated-measures analyses were performed for changes in FBF and FVV. In vitro data were analyzed with 2-way ANOVA.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Subject Characteristics

Grouped baseline characteristics for all subjects are shown in Table 1.

Protocol A: Normoxia

Venous Tone

At doses of nitrite of 784 nmol/min, 3.14 μmol/min, and 7.84 μmol/min, very large decreases in forearm venous tone were observed (Figure 1). The time course over which the dilation occurred is presented in Table 2 and Figure 2. Peak venodilation (mean±SEM) at 3.14 and 7.84 μmol/min was 20.6±4.2% ($P<0.05$) and 35.8±7.5% ($P<0.005$), respectively.

Arterial Blood Flow

Only at 3.14 and 7.84 μmol/min were there noticeable increases in FBF (Figure 3). At baseline, FBF was 1.0±0.1 (mean±SEM). At 3.14 and 7.84 μmol/min, FBF was 1.8±0.3 and 1.6±0.2, respectively ($P<0.05$).

Plasma Nitrite and Protein-Bound NO Levels

Venous plasma nitrite levels were significantly greater in the infused versus control arm at doses of ≥314 nmol/mL

Table 2. Venodilation at Doses of Sodium Nitrite at 5, 12, and 20 Minutes of Infusion

Dose of Nitrite	% Venodilation		
	5 min	12 min	20 min
40 nmol/min	-2.2±1.7	-2.8±2.7	-4.2±1.5
100 nmol/min	-5.1±2.6	-3.1±2.3	-0.8±4.6
314 nmol/min	1.6±3.1	8.2±4.0	8.1±4.3
784 nmol/min	4.0±2.5	7.3±2.7	10.3±2.8
3.14 μmol/min	17.6±4.1	19.1±3.3*	20.6±4.2*
7.84 μmol/min	29.1±10.1†	30.7±4.3‡	35.8±7.5‡

All values are presented as mean±SEM.
**P*<0.05, †*P*<0.005, and ‡*P*<0.001 vs baseline.

(*P*<0.05; Figure 4. No statistically significant differences were present in venous levels of plasma protein-bound NO between the 2 arms at any dose Figure 4).

Arterial levels of plasma protein-bound NO in the infused arm increased from 64.0±5.8 nmol/L at baseline to 83.8±23.8 nmol/L (Figure 5B; *P*=NS).

Protocol B: Hypoxia

Venous Tone and FBF

Venodilation was similar between normoxia and hypoxia; however, hypoxia significantly enhanced the increase in FBF during nitrite infusion. The mean increase in FBF during high-dose nitrite infusion (7.84 μmol/min) in hypoxia was significantly greater than that in normoxia (*P*<0.05; Figure 6). Low-dose nitrite (314 nmol/min) had little effect during normoxia but increased FBF during hypoxia (*P*<0.05; Figure 7).

In Vitro Work

Nitrite-induced vessel relaxation increased proportionally with increasing nitrite concentrations in both the aorta and the vena cava (*P*<0.05; Figure 8). No statistical difference was observed between the degree of nitrite-induced relaxation in the aorta versus vena cava under normoxia. Hypoxia enhanced nitrite-induced relaxation in both vessel types compared with relaxation under normoxia; however, veins had a proportionally greater relaxation than arteries at 100 and 1000 μmol/L nitrite (*P*<0.01).

Discussion

The present study showed that under normoxic conditions, exogenous nitrite-induced vasodilation in humans occurred predominantly in capacitance vessels. Under these conditions, nitrite only modestly increased FBF (×1.6), even at

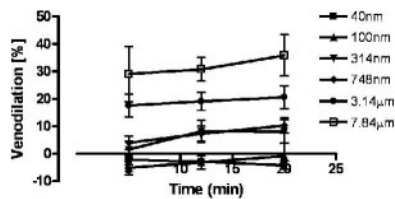


Figure 2. Venodilation measured at 5, 12, and 20 minutes during infusion of nitrite at each of the infusion doses during normoxia.

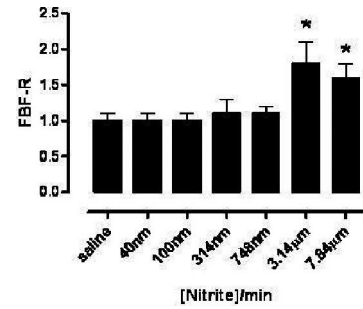


Figure 3. FBF at incremental doses of sodium nitrite under normoxic conditions. Data are expressed as a ratio of infused arm to control arm (FBF-R) to correct for any systemic changes. Values are mean±SEM. **P*<0.05.

maximal arterial nitrite concentrations (≈30 μmol/L). Although hypoxia had no incremental influence on the effects of nitrite on capacitance vessels, at similar concentrations (≈55 μmol/L), it had a profound relaxing effect on resistance vessels. The in vitro studies confirmed this influence of hypoxia on nitrite-induced relaxation in both arteries and veins, which suggests that oxygen tension is a major determinant of the degree of response to nitrite.

Until recently, the nitrite anion was regarded as a relatively inert byproduct of NO metabolism and even as a potentially carcinogenic source of *N*-nitroso compounds.³⁶ Recently, research has shown nitrite to be a distinct and important signaling molecule.⁸ Normoxic studies with animal aortic strips appeared to preclude a physiological role,⁴ but in contradistinction, a physiological function for nitrite has been supported by the capacity of physiological nitrite concentrations (≈1 to 2 μmol/L) to dilate human resistance vessels.⁹

We have now shown that exogenous nitrite is a potent dilator of the venous capacitance bed in humans under both normoxic and hypoxic conditions. At the peak dose, we observed ≈40% venodilation; this is a large effect. Because up to 70% of the circulating blood volume resides within the capacitance bed, even subtle modifications of venous tone

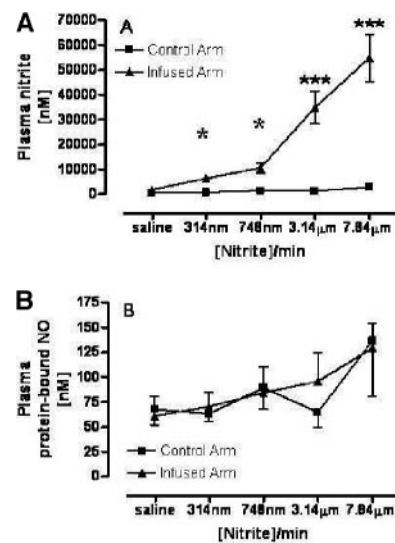


Figure 4. Plasma nitrite (A) and protein-bound NO (B) levels under normoxic conditions. All values are presented as mean±SEM. **P*<0.05 and ****P*<0.001 vs control arm.

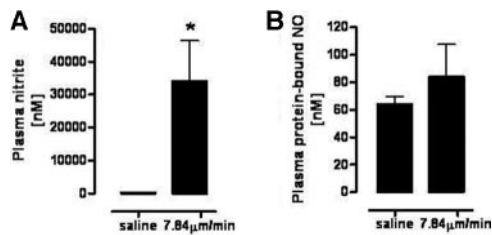


Figure 5. Arterial levels of plasma nitrite in the infused arm increased from 233 ± 27.0 nmol/L at baseline to 34.1 ± 12.3 μmol/L (Figure 5A; $P < 0.05$).

can result in large changes in central blood volume and cardiac “preload.” Accordingly, a strong selective venodilator may confer distinct benefits in the treatment of heart failure. We have previously used carbachol extensively in studies assessing venous capacitance.³⁷ At its peak dose, a maximal 3- to 4-fold increase in FBF and a 40% venodilation was observed.¹⁹ Thus, nitrite exerts venodilatory effects similar to those of carbachol with comparatively modest effects on resistance vessels during normoxia.

The enhanced resistance-vessel response to nitrite during hypoxia is consistent with the findings of Cosby et al,⁹ who observed that the vasodilatory effects of intra-arterial nitrite were enhanced by the tissue hypoxia induced by exercise of the forearm. Moreover, the present study answers many of the questions that were raised after publication of the study by Cosby et al⁹ and demonstrates in particular that hypoxia per se is a primary determinant in the action of nitrite. We found that in normoxia, low-dose nitrite (314 nmol/min) had no discernable effect on FBF, whereas Cosby et al⁹ observed a 29% increase in FBF in response to nitrite infused at 400

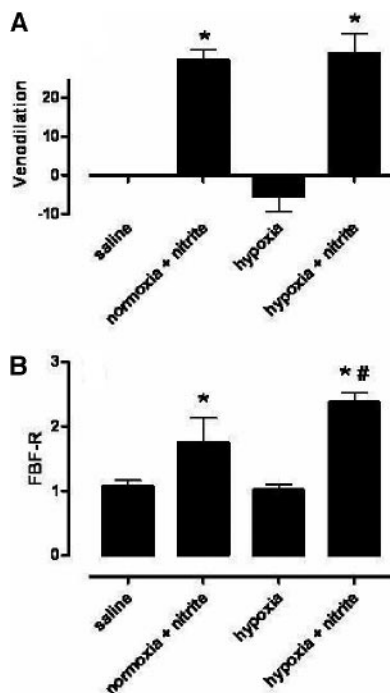


Figure 6. Effect of hypoxia on venodilation (A) and FBF-R (B) induced by intrabrachial nitrite (7.84 μmol · L⁻¹ · min⁻¹). Values are mean ± SEM. * $P < 0.05$ vs baseline; # $P < 0.05$ vs normoxia and nitrite.

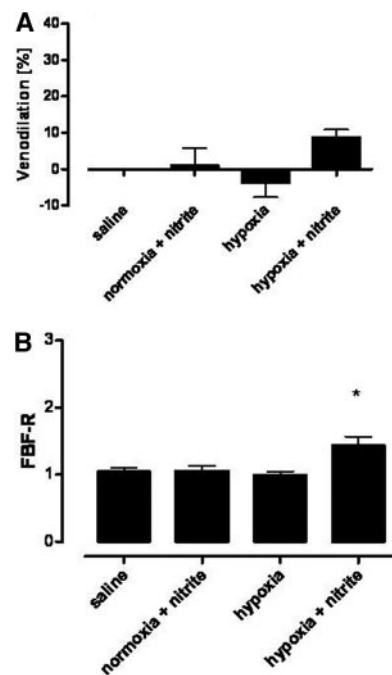


Figure 7. Effect of hypoxia on venodilation (A) and FBF-R (B) induced by intrabrachial nitrite (314 nmol · L⁻¹ · min⁻¹). Values are mean ± SEM. * $P < 0.05$ vs normoxia and nitrite.

nmol/min in normoxia, but Lauer et al⁵ reported that exogenous nitrite had no effect at all on FBF, even at doses as high as 36 μmol/min. The reasons for this discrepancy may pertain to differences in the subjects and the experimental protocols, although such differences are not obvious because the preparation of nitrite and its infusion were similar. It is unlikely that subjects in the present study differed significantly in sympathetic stimulation.³⁸ It is possible, however, that heterogeneity exists in the handling of nitrite. In the present study, we found a significant degree of variation in the responses observed. Although in the majority of individuals, nitrite doses as low as 314 nmol/min had little measurable effect on FBF, in a few subjects, the effect was more pronounced. In 1 individual, the FBF increased by 27%. Such heterogeneity may explain some of the apparently contradictory findings in the literature and provides an interesting avenue for future work.

Although in normoxia, nitrite was a weak vasodilator of resistance vessels compared with agents such as acetylcholine³⁹ and bradykinin⁴⁰ that can induce a 3- to 4-fold increase

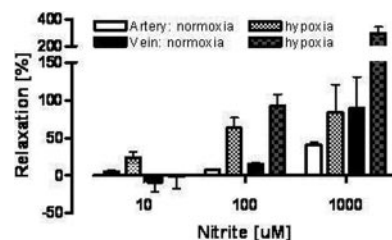


Figure 8. Relaxation of aorta and inferior vena cava after 20 minutes of incubation with varying nitrite concentrations under either normoxic or hypoxic conditions. Data are mean ± SEM. Each data set is n=4 to 5.

in FBF, in hypoxia, we show that it becomes an arterial vasodilator of comparable potency to these agents. Importantly, a significant augmentation in FBF during hypoxia was also seen with low-dose (314 nmol/min) nitrite, which implies a possible physiological role under hypoxic conditions.

Our vascular ring studies confirmed that in the absence of hemoglobin, nitrite-induced dilatation of veins and arteries is more profound in the context of hypoxia than in normoxia, which confirms the present findings *in vivo*. *In vitro*, during hypoxia, relaxation of veins was greater than that of arteries; however, it is not valid to directly compare potency between arteries and veins and extrapolate to the *in vivo* situation because of the nonphysiological nature of the preparation, which measures relaxation from a precontracted state rather than changes in basal tone.

Although the present study confirms a role for hypoxia in facilitating exogenous nitrite-induced vasodilation, no conclusion can be drawn about the mechanisms subserving this effect. Both nonenzymatic chemical reactions⁴¹ and nitrite reductase species⁴² as diverse as hemoglobin,^{9,30,43} xanthine oxidase,¹⁵ and endothelial NO synthase,⁴⁴ as well as the mitochondrial enzymes cytochrome *c* oxidase and the bc1 complex,⁴⁵ reduce nitrite hypoxically *in vitro*; however, their relative contribution to the hypoxic vasodilatory response *in vivo* remains unresolved.^{2,11,42} In subjects infused with nitrite, NO adducts of hemoglobin are increased, and it has been suggested that hemoglobin is a key nitrite reductase.⁹ Hemoglobin can subserve a number of roles, including nitrite reductase in hypoxia; as an acceptor of the resulting NO (eg, through HbNO); and as a donor of NO precisely where it is required, ie, at the site of hypoxia (through *S*-nitrosohemoglobin). These multiple personalities make hemoglobin an attractive nitrite modulator. A recent *in vitro* study⁴⁶ has observed that nitrite-dependent vasodilation conforms to first-order reaction kinetics with respect to nitrite concentration. Using selective inhibitors during hypoxia, that study indicated that nitrite-induced vasorelaxation was independent of the nitrite reductases listed above and importantly was inhibited by oxygenated hemoglobin but not by deoxygenated hemoglobin.⁴⁶ The study did not exclude a role for hemoglobin as a nitrite modulator *in vivo*. If nitrite is subject to hemoglobin-mediated NO production, how the resulting products or NO itself escapes from the red blood cell is not yet clear, because such an escape is difficult to reconcile with the diffusional limitations and the time constraints of an extremely quick A-to-V transit time.¹¹

To reconcile the site and mechanism of action of nitrite *in vivo*, vascular preparations will need to be investigated further. Specific agents and inhibitors are required to identify putative nitrite reductases, eg, hemoglobin, myoglobin, neuroglobin, and other heme proteins, such as hypoxia-dependent nitrite reductases.⁴⁷ The species to which nitrite is converted and which activate guanylate cyclase, eg, NO, iron nitrosyl, *N*-nitroso, and *S*-nitroso complexes, will also need to be identified. Parallel *in vivo* studies will supplement the *in vitro* studies. In the present study, venous plasma nitrosothiol levels rose similarly in both arms, which suggests that protein-bound NO in the plasma compartment did not medi-

ate the venous dilation directly. This, however, does not preclude protein-bound NO within the red blood cells or within the vascular compartment contributing to the bioactive effects of nitrite.

Study Limitations

The present study was performed in healthy volunteers, and the response to nitrite may differ in disease. In addition, organ-chamber bioassays are limited by the complicating factor of varying baseline tension in precontracted vessels.¹¹ Because different vessels (capacitance versus resistance) show different and nonlinear responses, such bioassay results should be assessed judiciously, and any interpretation of apparent differences between vessel types should be guarded. Nonetheless, the present *in vivo* and *in vitro* studies are consistent internally. Finally, the present results increase the understanding of the synergy between nitrite and hypoxia but provide limited insights into the role of endogenous nitrites in physiology. We speculate that the present study may also implicate a role for endogenous nitrites in hypoxic vessels, but we have limited any discussion of the role of nitrite in physiological hypoxic vasodilatation.¹¹ Even in the *in vitro* assays, in which unphysiologically high concentrations of nitrite (100 $\mu\text{mol/L}$) have been required previously, careful attention to precontraction and incipient conditions reveals that physiological concentrations of nitrite (0.1 to 10 $\mu\text{mol/L}$) can modulate tone.⁴⁶

Conclusions

The present study confirms that hypoxia per se is a prime determinant of the *in vivo* response to exogenous nitrite. Both capacitance and resistance vessels respond profoundly to relatively modest pharmacological concentrations of exogenous nitrite in hypoxia. Although for some years, it has been speculated on the basis of studies in normoxic and exercising patients that "therapeutic application of nitrite should result in selective vasodilation to hypoxemic tissue, and could be used to treat diseases associated with ischemia,"⁹ the present study confirms the role for nitrite in resistance-vessel dilatation in human hypoxic tissues. Corresponding therapeutic implications exist in conditions such as heart failure, in which selective venodilation would reduce intracardiac pressure in the absence of resistance-vessel dilatation, thus avoiding hypotension. Perhaps more importantly, pharmacological doses of nitrite may constitute a specific hypoxically bioactive agent that targets only hypoxic vessels.^{12,48,49} A preliminary indication of the importance of nitrite has been afforded by data presented by Arai,² who demonstrated that a brief nitrite infusion (plasma concentration of $\approx 5 \mu\text{mol/L}$) decreased myocardial infarction size from 70% to 20%. In addition to confirming the present findings, future studies will have to assess the therapeutic role of nitrite in disease.

Acknowledgments

Special thanks to Bill Thomson, Joe O'Brien, and the radiopharmacy team for their medical physics and radiopharmaceutical support. We also acknowledge Janis Weeks (Medical Microbiology, Cardiff) for conducting the plasma nitrite/protein NO measurements and for her expertise in the biochemical aspects of this study.

Sources of Funding

This research was supported by the British Heart Foundation.

Disclosures

Dr Frenneaux has received a research grant from Medtronic. He has an ownership interest in a "method of use" patent held for Perhexiline in Chronic Heart Failure. He has also served on the advisory board or as a consultant to Medtronic and Biotronic and has received sponsorship from Medtronic, Biotronic, Servier, Novartis, and AstraZeneca to attend meetings.

References

- Segal SS, Duling BR. Flow control among microvessels coordinated by intercellular conduction. *Science*. 1986;234:868–870.
- Arai A. Low-dose nitrite ameliorates myocardial ischemia/reperfusion injury and reduces infarct size in a canine model. Presented at: Role of Nitrite in Physiology, Pathophysiology, and Therapeutics Meeting; September 8–9, 2005; Bethesda, Md. Cited by Gladwin MT, Raat NJ, Shiva S, Dezfulian C, Hogg N, Kim-Shapiro DB, Patel RP. Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. *Am J Physiol Heart Circ Physiol*. 2006;291:H2026–H2035.
- Segal SS. Regulation of blood flow in the microcirculation. *Microcirculation*. 2005;12:33–45.
- Furchgott RF, Bhadrakom S. Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs. *J Pharmacol Exp Ther*. 1953;108:129–143.
- Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, Kelm M. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. *Proc Natl Acad Sci U S A*. 2001;98:12814–12819.
- Singel DJ, Stamler JS. Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin. *Annu Rev Physiol*. 2005;67:99–145.
- Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, Maloney RE, Bharti A, Rodriguez J, Feelisch M. Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat Chem Biol*. 2005;1:290–297.
- Bryan NS. Nitrite in nitric oxide biology: cause or consequence? A systems-based review. *Free Radic Biol Med*. 2006;41:691–701.
- Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO III, Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med*. 2003;9:1498–1505.
- Gladwin MT, Shelhamer JH, Schechter AN, Pease-Fye ME, Waclawiw MA, Panza JA, Ognibene FP, Cannon RO III. Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. *Proc Natl Acad Sci U S A*. 2000;97:11482–11487.
- Luchsinger BP, Rich EN, Yan Y, Williams EM, Stamler JS, Singel DJ. Assessments of the chemistry and vasodilatory activity of nitrite with hemoglobin under physiologically relevant conditions. *J Inorg Biochem*. 2005;99:912–921.
- Duranski MR, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT, Lefer DJ. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J Clin Invest*. 2005;115:1232–1240.
- Jung KH, Chu K, Ko SY, Lee ST, Sinn DI, Park DK, Kim JM, Song EC, Kim M, Roh JK. Early intravenous infusion of sodium nitrite protects brain against in vivo ischemia-reperfusion injury. *Stroke*. 2006;37:2744–2750.
- Tripatara P, Patel NS, Webb A, Rathod K, Lecomte FM, Mazzon E, Cuzzocrea S, Yaqoob MM, Ahluwalia A, Thiemermann C. Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: role for xanthine oxidoreductase. *J Am Soc Nephrol*. 2007;18:570–580.
- Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc Natl Acad Sci U S A*. 2004;101:13683–13688.
- Beier S, Classen HG, Loeffler K, Schumacher E, Thoni H. Antihypertensive effect of oral nitrite uptake in the spontaneously hypertensive rat. *Arzneimittelforschung*. 1995;45:258–261.
- Dias-Junior CA, Gladwin MT, Tanus-Santos JE. Low-dose intravenous nitrite improves hemodynamics in a canine model of acute pulmonary thromboembolism. *Free Radic Biol Med*. 2006;41:1764–1770.
- Pluta RM, Dejam A, Grimes G, Gladwin MT, Oldfield EH. Nitrite infusions to prevent delayed cerebral vasospasm in a primate model of subarachnoid hemorrhage. *JAMA*. 2005;293:1477–1484.
- Blackman DJ, Morris-Thurgood JA, Atherton JJ, Ellis GR, Anderson RA, Cockcroft JR, Frenneaux MP. Endothelium-derived nitric oxide contributes to the regulation of venous tone in humans. *Circulation*. 2000;101:165–170.
- Schmitt M, Blackman DJ, Middleton GW, Cockcroft JR, Frenneaux MP. Assessment of venous capacitance: radionuclide plethysmography: methodology and research applications. *Br J Clin Pharmacol*. 2002;54:565–576.
- Clements IP, Strelow DA, Becker GP, Vlietstra RE, Brown ML. Radionuclide evaluation of peripheral circulatory dynamics: new clinical application of blood pool scintigraphy for measuring limb venous volume, capacity, and blood flow. *Am Heart J*. 1981;102:980–983.
- Manyari DE, Malkinson TJ, Robinson V, Smith ER, Cooper KE. Acute changes in forearm venous volume and tone using radionuclide plethysmography. *Am J Physiol*. 1988;255:H947–H952.
- Rogers SC, Khalatbari A, Gapper PW, Frenneaux MP, James PE. Detection of human red blood cell-bound nitric oxide. *J Biol Chem*. 2005;280:26720–26728.
- Feelisch M, Rassaf T, Mnaimneh S, Singh N, Bryan NS, Jourdain D, Kelms M. Concomitant S-, N-, and heme-nitrosylation in biological tissues and fluids: implications for the fate of NO in vivo. *FASEB J*. 2002;16:1775–1785.
- Rassaf T, Preik M, Kleinbongard P, Lauer T, Heiss C, Strauer BE, Feelisch M, Kelm M. Evidence for in vivo transport of bioactive nitric oxide in human plasma. *J Clin Invest*. 2002;109:1241–1248.
- Xu X, Cho M, Spencer NY, Patel N, Huang Z, Shields H, King SB, Gladwin MT, Hogg N, Kim-Shapiro DB. Measurements of nitric oxide on the heme iron and beta-93 thiol of human hemoglobin during cycles of oxygenation and deoxygenation. *Proc Natl Acad Sci U S A*. 2003;100:11303–11308.
- Yang BK, Vivas EX, Reiter CD, Gladwin MT. Methodologies for the sensitive and specific measurement of S-nitrosothiols, iron-nitrosyls, and nitrite in biological samples. *Free Radic Res*. 2003;37:1–10.
- Hausladen A, Rafikov R, Angelo M, Singel DJ, Nudler E, Stamler JS. Assessment of nitric oxide signals by triiodide chemiluminescence. *Proc Natl Acad Sci U S A*. 2007;104:2157–2162.
- MacArthur PH, Shiva S, Gladwin MT. Measurement of circulating nitrite and S-nitrosothiols by reductive chemiluminescence. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;851:93–105.
- Nagababu E, Ramasamy S, Abernethy DR, Rifkind JM. Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction. *J Biol Chem*. 2003;278:46349–46356.
- Gladwin MT, Bryan NS, Maloney RE, Specian V, Kelm M, Kalyanaraman B, Rodriguez J, Feelisch M. NO adducts in mammalian red blood cells: too much or too little? *Nat Med*. 2003;9:481–482.
- Stamler JS. S-nitrosothiols in the blood: roles, amounts, and methods of analysis. *Circ Res*. 2004;94:414–417.
- Wang X, Bryan NS, MacArthur PH, Rodriguez J, Gladwin MT, Feelisch M. Measurement of nitric oxide levels in the red cell: validation of tri-iodide-based chemiluminescence with acid-sulfanilamide pretreatment. *J Biol Chem*. 2006;281:26994–27002.
- Rogers SC, Khalatbari A, Datta BN, Ellery S, Paul V, Frenneaux MP, James PE. NO metabolite flux across the human coronary circulation. *Cardiovasc Res*. 2007;75:434–441.
- Gunaruwan P, Schmitt M, Taylor J, Lee L, Struthers A, Frenneaux M. Lack of rapid aldosterone effects on forearm resistance vasculature in health. *J Renin Angiotensin Aldosterone Syst*. 2002;3:123–125.
- Gladwin MT. Nitrite as an intrinsic signaling molecule. *Nat Chem Biol*. 2005;1:245–246.
- Nightingale AK, Blackman DJ, Ellis GR, Schmitt M, Morris-Thurgood JA, Jones EA, Frenneaux MP. Preservation of venous endothelial function in the forearm venous capacitance bed of patients with chronic heart failure despite arterial endothelial dysfunction. *J Am Coll Cardiol*. 2001;37:1062–1068.
- Weisbrod CJ, Minson CT, Joyner MJ, Halliwill JR. Effects of regional phenolamine on hypoxic vasodilatation in healthy humans. *J Physiol*. 2001;537:613–621.
- Larrousse M, Bragulat E, Segarra M, Sierra C, Coca A, de La Sierra A. Increased levels of atherosclerosis markers in salt-sensitive hypertension. *Am J Hypertens*. 2006;19:87–93.

40. Wotherspoon F, Browne DL, Meeking DR, Allard SE, Munday LJ, Shaw KM, Cummings MH. The contribution of nitric oxide and vasodilatory prostanooids to bradykinin-mediated vasodilation in type 1 diabetes. *Diabet Med*. 2005;22:697–702.
41. Zweier JL, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. *Nat Med*. 1995;1:804–809.
42. Lundberg JO, Weitzberg E. NO generation from nitrite and its role in vascular control. *Arterioscler Thromb Vasc Biol*. 2005;25:915–922.
43. Doyle MP, Pickering RA, DeWeert TM, Hoekstra JW, Pater D. Kinetics and mechanism of the oxidation of human deoxyhemoglobin by nitrites. *J Biol Chem*. 1981;256:12393–12398.
44. Gautier C, van Faassen E, Mikula I, Martasek P, Slama-Schwok A. Endothelial nitric oxide synthase reduces nitrite anions to NO under anoxia. *Biochem Biophys Res Commun*. 2006;341:816–821.
45. Kozlov AV, Staniek K, Nohl H. Nitrite reductase activity is a novel function of mammalian mitochondria. *FEBS Lett*. 1999;454:127–130.
46. Dalsgaard T, Simonsen U, Fago A. Nitrite-dependent vasodilation is facilitated by hypoxia and is independent of known NO-generating nitrite reductase activities. *Am J Physiol Heart Circ Physiol*. 2007;292:H3072–H3078.
47. Huang Z, Shiva S, Kim-Shapiro DB, Patel RP, Ringwood LA, Irby CE, Huang KT, Ho C, Hogg N, Schechter AN, Gladwin MT. Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. *J Clin Invest*. 2005;115:2099–2107.
48. Kim-Shapiro DB, Schechter AN, Gladwin MT. Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. *Arterioscler Thromb Vasc Biol*. 2006;26:697–705.
49. Lefer DJ. Nitrite therapy for protection against ischemia-reperfusion injury. *Am J Physiol Renal Physiol*. 2006;290:F777–F778.

CLINICAL PERSPECTIVE

Advances in cardiovascular treatment could be made through the understanding and utilization of nitrite as a stable store of transported nitric oxide, allowing the targeted delivery of nitric oxide precisely where it is needed, both physiologically and therapeutically. Data are presented that demonstrate that nitrite-induced vasodilation in humans is a function of ambient oxygen tension. Subjects received intrabrachial sodium nitrite while breathing either room air or 12% oxygen. During normoxia, the nitrite infusion had modest effects in resistance vessels but caused a significant vasodilation in the relatively hypoxic capacitance bed. However, when the oxygen tension in the resistance vessels decreased because the subjects were breathing 12% oxygen, nitrite caused a marked dilatation in this bed. Nitrite has been shown to decrease infarct size by up to 50% in a canine model of myocardial infarction. Studies are under way to ascertain whether nitrite is as potent in reducing myocardial damage in humans. Similarly, nitrite has been shown to mitigate ischemia-reperfusion injury of the brain, kidney, and liver. The present findings suggest that nitrite may play a role in the physiological defense against ischemia. This mechanism lends itself to therapeutic exploitation in clinical scenarios such as acute coronary syndromes in which critical ischemia is the primary underlying pathology. This mechanism potentially may also be used in the treatment of acute decompensated heart failure. The use of a selective venodilator could avoid the deleterious hypotensive effects of current vasodilators that cause a significant degree of arterial vasodilation.