Enhanced Vasodilator Activity of Nitrite in Hypertension : Critical Role for Erythrocytic Xanthine Oxidoreductase and Translational Potential

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Critical Role for Erythrocytic Xanthine Oxidoreductase and Translational Potential

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Abstract—Elevation of circulating nitrite (NO_2^-) levels causes vasodilatation and lowers blood pressure in healthy volunteers. Whether these effects and the underpinning mechanisms persist in hypertension is unknown. Therefore, we investigated the consequences of systemic nitrite elevation in spontaneously hypertensive rats and conducted proof-of-principle studies in patients. Nitrite caused dose-dependent blood pressure–lowering that was profoundly enhanced in spontaneously hypertensive rats versus normotensive Wistar Kyoto controls. This effect was virtually abolished by the xanthine oxidoreductase (XOR) inhibitor, allopurinol, and associated with hypertension-specific XOR-dependent nitrite reductase activity localized to the erythrocyte but not the blood vessel wall. To determine whether these pathways translate to human hypertension, we investigated the effects of elevation of circulating nitrite levels in 15 drug naïve grade 1 hypertensives. To elevate nitrite, we used a dose of dietary nitrate (≈3.5 mmol) that elevated nitrite levels ≈1.5-fold (*P*<0.01); a rise shown previously to exert no significant blood pressure–lowering effects in normotensives. This dose caused substantial reductions in systolic (≈12 mm Hg) and diastolic blood pressures (*P*<0.001) and pulse wave velocity (*P*<0.05); effects associated with elevations in erythrocytic XOR expression and XOR-dependent nitrite reductase activity. Our observations demonstrate the improved efficacy of inorganic nitrate and nitrite in hypertension as a consequence of increased erythrocytic XOR nitrite reductase activity and support the concept of dietary nitrate supplementation as an effective, but simple and inexpensive, antihypertensive strategy. (***Hypertension.*** **2013;61:1091-1102.**) ● **Online Data Supplement**

Key Words: hypertension ■ nitric oxide ■ red blood cells ■ vascular biology

Despite the substantial advances made in antihypertensive pharmacotherapy, it is estimated that by 2025 there will be 1.5 billion people with hypertension worldwide.[1] Indeed, over the past 3 decades the number of patients with uncontrolled essential hypertension has continued to rise year by year.[2] This imperative has renewed interest in attempting to harness the potential beneficial effects of diets rich in fruit and vegetables.[3] This focus, in part, emanates from a perception that dietary interventions may be more acceptable and achievable for many patients. Such an option carries greater resonance when one considers that blood pressure (BP) remains elevated in ≈30% to 40% of all treated hypertensives.[4] Although some of these individuals likely have what is termed "resistant hypertension" (recent estimates suggest ≈9% in the United States),[5] for many of these individuals compliance likely underlies this phenomenon.[6]

Recently, it has been proposed that the inorganic nitrate (NO_3^-) content of a healthy diet may underlie the beneficial effects of a fruit- and vegetable-rich diet. Inorganic nitrate is present in most vegetables and is especially abundant in green leafy vegetables and beetroot (*Beta vulgaris*). Small-scale clinical studies demonstrate that orally-ingested inorganic nitrate is sequentially bioactivated, within the enterosalivary circuit, to inorganic nitrite (NO_2^-)[7] which is then, in turn, converted to the signaling molecule nitric oxide (NO) within the systemic circulation. Although the reduction of nitrate to nitrite is not a response of the host, but rather a reaction facilitated by facultative bacteria that have colonized the dorsal surface of the tongue,[8] the reduction of nitrite to NO is a host response. Within the blood vessel, nitrite reduction is thought to be facilitated by several enzyme-dependent and -independent nitrite reductase pathways.[9] NO, in turn, exerts several beneficial effects within

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the circulation, including the classical vasodilator NO-signaling cascade involving activation of soluble guanylate cyclase and elevation of cyclic GMP (cGMP).[10,11]

Accordingly, systemic nitrite administration causes dose-dependent decreases in BP in both healthy animals and volunteers.[12–15] In addition, chronic oral administration of nitrite reduces BP without any evidence of tachyphylaxis in genetic, surgical, and salt-induced animal models of hypertension,[16–20] although in general the doses of nitrite tested substantially exceed physiological levels. However, the vascular nitrite reductase that underpins these BP-lowering effects has not been established. This uncertainty stems from the fact that nitrite reduction is a phenomenon that increases with decreasing pH and oxygen tension.[21] These conditions simultaneously upregulate and favor the activity of several vascular nitrite reductases, including xanthine oxidoreductase (XOR), deoxyhemoglobin, aldehyde oxidase, and even endothelial NO synthase (eNOS).[22] Because the BP-lowering effects of nitrite occur under physiological conditions, there is considerable uncertainty regarding both the identity of the key nitrite reductase as well as its cellular localization. Within the literature, there is evidence suggesting that this activity is localized to the erythrocyte and the blood vessel wall.[15,23,24] Whether the oxidative environment in the vasculature of hypertensive patients might alter the site, pathway, and extent of nitrite reductase activity is unknown.

In this study, we have explored whether nitrite-induced BP-lowering activity might be enhanced in hypertension and the mechanisms involved in any beneficial effects seen in an animal model of hypertension and in hypertensive individuals.

Methods
For expanded methods, please see online-only Data Supplement.

Animal Studies
All experiments were conducted according to the Animals (Scientific Procedures) Act 1986, United Kingdom and approved by the UK Home Office. Male spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) controls (14–16 weeks of age, Charles River, United Kingdom) were used for in vivo BP measurements or blood and tissue collection for in vitro studies. All drugs used were purchased form Sigma Aldrich, United Kingdom, unless specified.

BP Measurement in SHR and WKY Rats
Rats were anesthetized and vessels cannulated for assessment of mean arterial pressure recorded in response to bolus doses of KNO_2 ($1–30 000 \times 10^{-9}$ mol/kg, 1 mL/kg, equivalent to $\approx 1 \times 10^{-8}$ to 3×10^{-4} mol/L circulating concentrations) and sodium nitroprusside (SNP, 1×10^{-5} g/kg). Some SHRs were pretreated with allopurinol (50 mg/kg, IV) or vehicle (0.1 mol/L NaOH, IV), and some WKY rats were given a constant infusion of phenylephrine (2 mg/kg per hour, IV) or saline to raise BP artificially.

Organ Bath and Tension Myography
Relaxation-response curves to KNO_2 ($0.01–30 \times 10^{-3}$ mol/L,) or spermine-NO ($0.001–3 \times 10^{-6}$ mol/L, Cambridge Biosciences, United Kingdom) were constructed in precontracted aortic rings or third order mesenteric resistance arteries in the absence or presence (30 minutes pretreatment) of the XOR inhibitor allopurinol (1×10^{-4} mol/L); the eNOS inhibitor, L-nitro monomethyl arginine (L-NMMA; 3×10^{-4} mol/L); the selective soluble guanylate cyclase inhibitor, 1H-[1,2,4] oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ; 1×10^{-6} mol/L); or the aldehyde oxidase inhibitor raloxifene, (1×10^{-5} mol/L).

Western Blotting
Equal amounts of protein of homogenates of aorta, mesentery, erythrocytes, or liver (80–100 µg) were subjected to western blotting to determine XOR expression levels.

Patient Study
The study was granted full ethical approval by the East London Research Ethics Committee. To elevate circulating nitrite levels noninvasively, volunteers were randomized to receive a dietary nitrate dose or placebo control. It has been demonstrated that the consumption of a single dose of dietary nitrate, in the form of inorganic nitrate capsules[25] or via the diet using nitrate-rich beetroot juice[25,26] or green leafy vegetables, results in an elevation of both circulating nitrate and nitrite levels.

In a proof-of-principle study, grade 1 drug-naïve hypertensive subjects (determined from previous 24-hour ambulatory BP measurements conducted <1 month before study date) were randomized in an open-label crossover design to receive 250 mL of inorganic nitrate-rich beetroot juice (dietary nitrate; James White Drinks, United Kingdom) or an equal volume of water (placebo; Zepbrook Ltd, United Kingdom). On each visit clinic BP measurements were made for 1 hour to provide baseline BP before intervention. Clinic BP and pulse wave velocity (PWV) were measured at specific time-points over the following 24-hour post intervention. Blood, urine, and saliva samples were collected for further biochemical analyses. This study is registered with clinicaltrials.gov (NCT01236872).

Measurement of Nitrate, Nitrite, and [cGMP] Levels
Plasma, tissue, and erythrocyte nitrite and nitrate levels were measured by ozone-based chemiluminescence[27] and cGMP determined using commercially available immunoassay.

Measurement of Nitrite Reductase Activity
The nitrite reductase activity of tissue supernatants and purified erythrocytes were determined using gas-phase chemiluminesence[28] at pH 7.4 (representing physiological conditions) or pH 6.8 (severe acidosis but also conditions that favor nitrite reductase pathways). Involvement of XOR was ascertained by pretreatment with allopurinol (1×10^{-4} mol/L) or vehicle for 30 minutes.

Results

Nitrite Exerts XOR-dependent Potent Hypotensive Effects in SHRs but not WKY Controls
As expected, SHRs at ≈ 16 weeks of age were hypertensive compared with WKY strain controls (Table 1). Nitrite exerted potent dose-dependent decreases in BP in SHR, but not WKY rats (Figure 1A), with significant decreases in BP first evident in SHRs, at doses of nitrite ($0.3–1 \times 10^{-6}$ mol/kg) that achieve estimated circulating concentrations of ≈ 1 to 3×10^{-6} mol/L

Table 1. Baseline Hemodynamic Parameters in SHR and WKY Rats

Parameter	WKY (n=12)	SHR (n=16)	WKY+PE (n=6)	P
SBP, mm Hg	119.3±2.6	153.4±3.9***	163.3±10.6***	<0.0001
DBP, mm Hg	74.4±3.8	101.1±2.7***	103.2±7.6***	<0.0001
MAP, mm Hg	95.6±3.6	126.8±3.3***	130.0±7.7***	<0.0001
HR, bpm	394.8±14.6	329.4±5.4***	361.8±18.7	0.004

Data are shown as mean±SE. Mean with statistical significance determined by 1-way ANOVA with ***$P<0.001$ vs WKY by Bonferroni post hoc analysis.

bpm indicates beats per minute; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; PE, phenylephrine; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; and WKY, Wistar Kyoto.
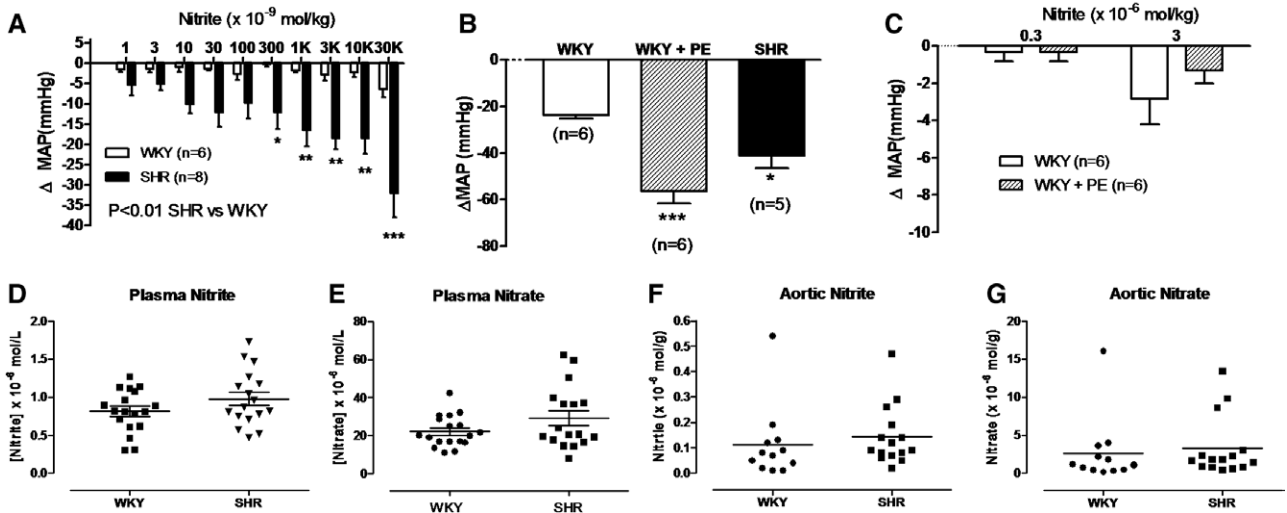
Figure 1. Nitrite-induced hypotension is greater in hypertensive rats. Changes in mean arterial blood pressure (MAP) in response to (**A**) KNO$_2$ and (**B**) nitric oxide donor, sodium nitroprusside (SNP, 10 µg/kg) in anesthetized normotensive Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats and (**C**) WKY rats with artificially raised blood pressure due to phenylephrine infusion (WKY+PE, 2 mg/kg per h). Levels of nitrite and nitrate in plasma (n=17–18; **D**, **E**) and aortic homogenates (n=12–15; **F**, **G**) of SHR and WKY control. Data shown as mean±SEM with n indicated on graphs. Significance values for 2-way or 1-way ANOVA followed by Bonferroni post hoc tests shown as *$P<0.05$, **$P<0.01$, and ***$P<0.001$ for SHR vs WKY.

(ie, within the physiological realm). At the highest doses-tested KNO$_2$, achieving a calculated circulating concentration of 3×10^{-4} mol/L nitrite caused a decrease in systolic BP (SBP) of 6.3±1.9 and 32.0±6.0 mm Hg in WKY and SHR animals, respectively. This decrease in SBP was specific to nitrite and not attributed to the K$^+$ in the salt because an equivalent dose of KCl caused decreases in SBP of 1.0±0.6 and 5.0±2.5 mm Hg, respectively (n=5 for each; $P>0.05$).

This increased sensitivity to nitrite, in SHR, was likewise exhibited to SNP (Figure 1B). Infusion of the constrictor

phenylephrine in WKY rats raised BP to levels comparable to baseline BP in SHR (Table 1). Under these conditions, the hypotensive response to SNP was substantially increased to a level matching the magnitude of the responses in SHR (Figure 1B). In contrast, the response to nitrite was unchanged (Figure 1C), suggesting that the increased BP in SHR per se did not account for the enhanced sensitivity to nitrite. There were no differences in baseline tissue or circulating levels of nitrite or nitrate between the strains (Figure 1D–1G).
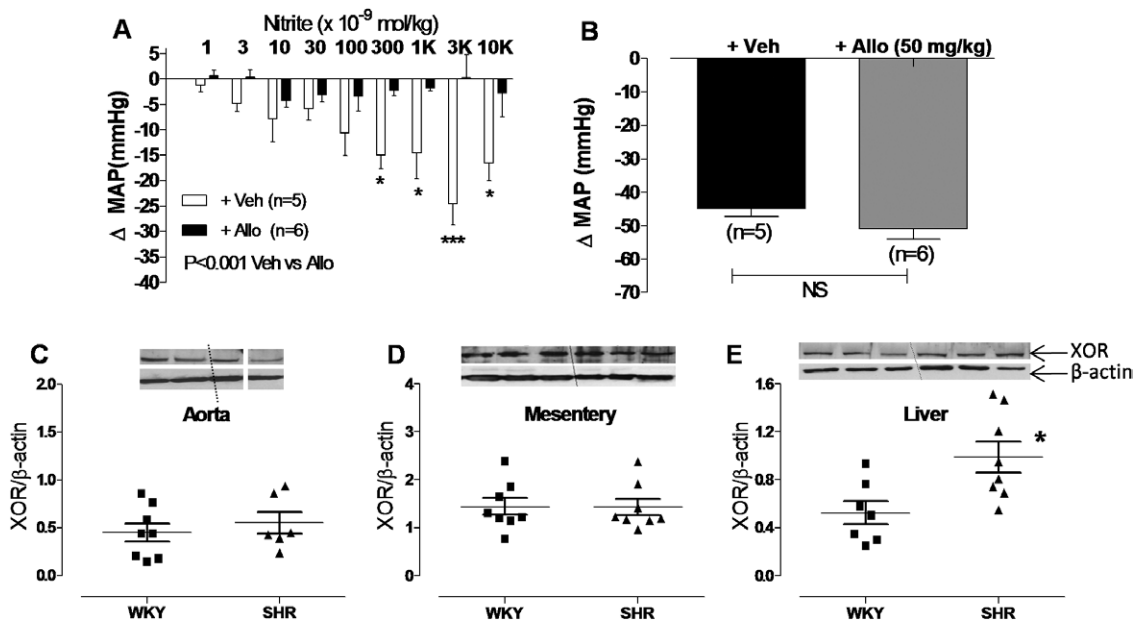
Figure 2. Xanthine oxidoreductase (XOR) mediates nitrite-induced decreases in blood pressure in spontaneously hypertensive (SHRs) rats. Changes in mean arterial blood pressure (MAP) in response to (**A**) KNO$_2$ or (**B**) nitric oxide donor, sodium nitroprusside (SNP, 10 µg/kg) in SHR treated with vehicle (veh, NaOH 0.1%) or XOR inhibitor, allopurinol (Allo, 50 mg/kg). XOR expression levels assessed by Western blotting of homogenates of aorta (**C**), mesenteric resistance arteries (**D**), and liver (**E**) of SHR and control Wistar Kyoto (WKY) rats. Significant difference between strains assessed using unpaired *t* tests.

Hypotensive Effects of Nitrite in SHR Are XOR-dependent

We next explored the possibility that XOR might be the nitrite reductase underlying nitrite bioactivity in the SHR. Indeed, allopurinol treatment profoundly suppressed the hypotensive effect of nitrite (Figure 2A) but had no effect on the response to SNP, in SHR (Figure 2B). There were no differences in XOR expression between the strains in either conduit and resistance arteries; however, XOR expression was elevated in SHR at one of the major sites for XOR synthesis, the liver (Figure 2C–2E; Figure S1A).

Enhanced Nitrite Reduction In Vivo in SHRs Is not Occurring at the Blood Vessel Wall

To determine whether the improved bioactivity of nitrite in SHRs related to enhanced nitrite activity at the level of the blood vessel wall, we investigated the vasorelaxant effects of nitrite in vitro. Both nitrite and spermine-NO produced concentration-dependent relaxation-response curves that were shifted to the right in arteries of SHR compared with WKY (Figure 3A–3D). This reduced activity of nitrite in arteries of SHRs was not attributed to a reduced nitrite reductase capacity because nitrite-derived NO formation was similar in blood vessel homogenates of SHR and WKY (Figure 3E and 3F). In addition, XOR inhibition

had no effect on relaxant responses to nitrite in any blood vessel tested (Figure S1A–S1D), although responses were abolished by ODQ (Figure S2E and S2F). Additionally, although raloxifene had no effect (Figure S2), L-NMMA and endothelial denudation enhanced nitrite-induced relaxation in both resistance arteries and aortic rings of SHRs (Figure S2). These results suggest that nitrite reduction at the blood vessel wall does not account for the BP-reducing effects in vivo.

Erythrocytic Nitrite Reductase Activity Is Enhanced in Experimental Hypertension

Because the erythrocyte has been proposed as an important site for nitrite reduction within the circulation, we assessed whether the nitrite reductase capacity of SHR erythrocytes might underlie the effects in vivo. Indeed, erythrocytic nitrite reductase activity of SHRs was substantially enhanced compared with WKY at pH 7.4 (Figure 4A and 4B). Furthermore, allopurinol near-abolished this activity. In contrast, no effect of allopurinol was evident in erythrocytes of WKY rats (Figure 4C–4F). Interestingly, erythrocyte levels of nitrite and nitrate were significantly lower in SHR compared with WKY (Figure 4G and 4H). This enhanced activity was associated with a near-doubling of erythrocytic XOR expression (Figure 4I).
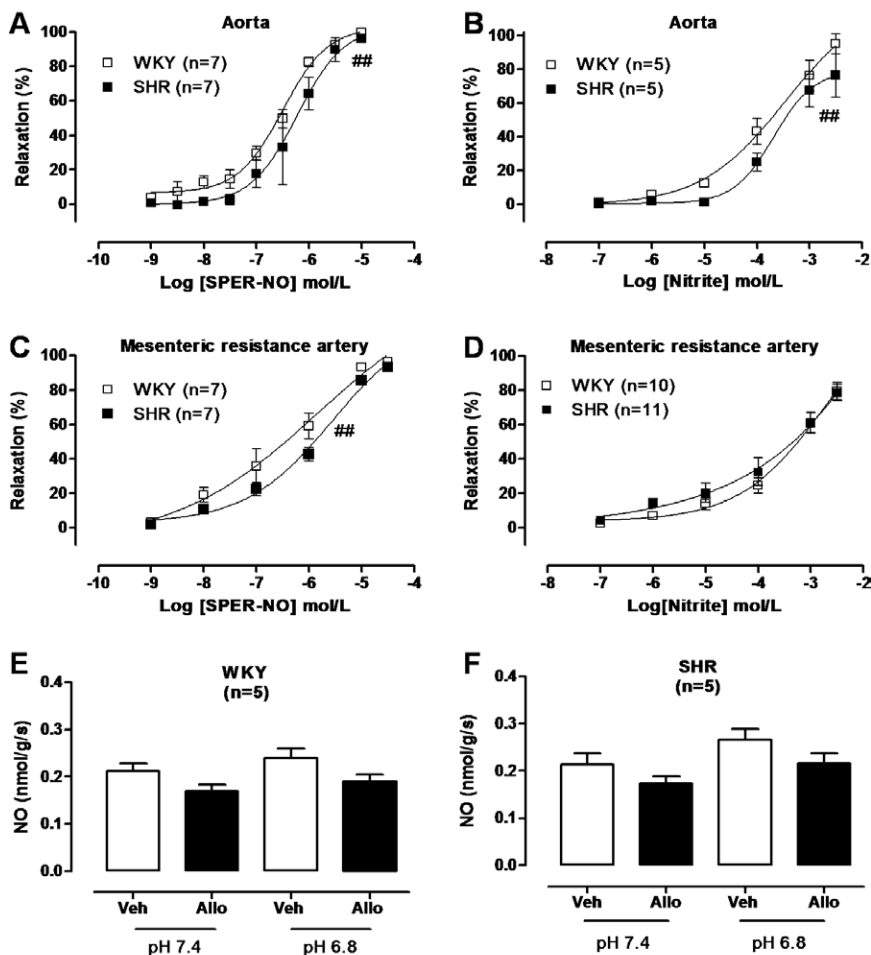
Figure 3. Nitrite bioactivity in resistance and conduit arteries of spontaneously hypertensive (SHR) compared with Wistar Kyoto (WKY) rats. Relaxation-response curves to nitrite and Spermine-NO (SPER-NO, n=7–11) in aortic rings (**A**, **B**) and mesenteric resistance arteries (**C**, **D**) of SHR and WKY rats. Nitrite reductase activity of aortic homogenates of SHR and WKY at pH 7.4 (**E**) and 6.8 (**F**) in the presence of nitrite (300 μmol/L). Data shown as mean±SEM with n indicated on graphs. Significance between curves determined using 2-way ANOVA and shown as ##*P*<0.01. No significant differences detected in nitrite reductase activity using 1-way ANOVA.

Figure 4. Xanthine oxidoreductase (XOR)-dependent nitrite reductase activity of rat erythrocytes. Comparison of the nitrite reductase activity of erythrocytes, purified from the blood of Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) animals, at pH 7.4 and pH 6.8 and in the presence of vehicle (veh) or the XOR inhibitor allopurinol (Allo, 100 μmol/L; **A–F**). Nitrite (**G**) and nitrate (**H**) concentrations of lysed erythrocytes purified from the blood of both WKY and SHR animal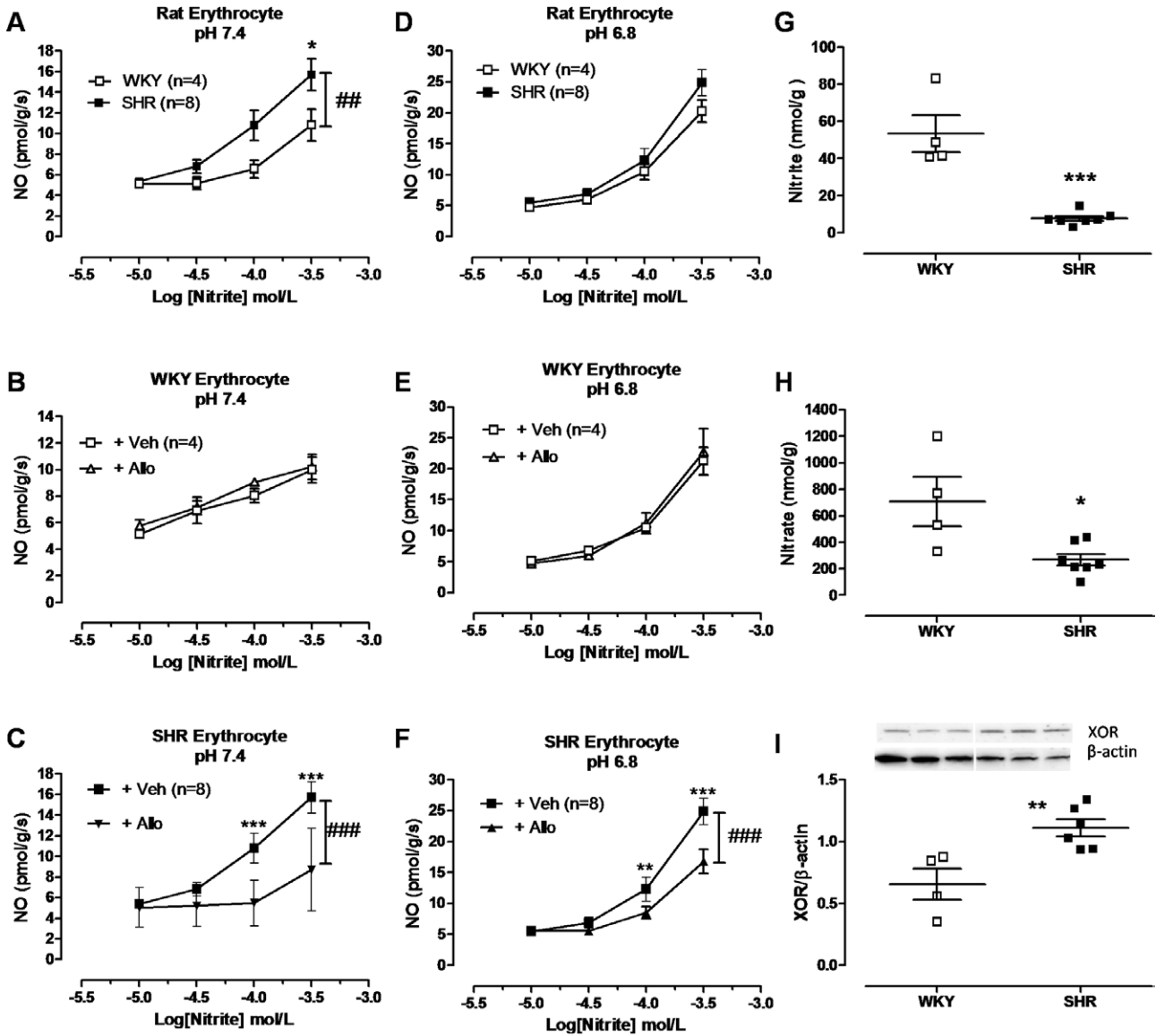s. **I**, Expression of erythrocytic XOR determined by Western blotting. Data are expressed as mean±SEM with n indicated on graphs. Statistical significance between curves determined using 2-way ANOVA and shown as ##P<0.01 and ###P<0.001 followed by Bonferroni post tests shown as *P<0.05, **P<0.01 and ***P<0.001. Comparison between 2 groups using unpaired t tests and shown as *P<0.05 and **P<0.01.

Nitrite Lowers BP and Improves Vascular Compliance in Hypertensive Patients

All individuals recruited were classified on a screening visit as having grade 1 hypertension (ie, SBP 140–159 or diastolic BP [DBP] 90–99 mm Hg) using 24-hour ambulatory BP measurement (Table 2).[29]

The intervention limb was composed of a dose of dietary nitrate of ≈3.5×10^{-3} mol nitrate in 250 mL of beetroot juice, that is, 13.2±0.94×10^{-3} mol/L (n=15). This dose sits just below the threshold dose of nitrate (4×10^{-3} mol) required to produce BP-lowering effects determined in dose-ranging studies in healthy, normotensive volunteers.[25] The placebo control was a matched volume of low-nitrate containing water (0.07±0.01×10^{-3} mol/L, n=15). The [nitrite] was <5×10^{-8} mol/L in both dietary nitrate and placebo interventions. There were no

significant differences in the general characteristics of volunteers between the limbs of the study (Table 3), which included 8 women and 7 men. Dietary nitrate, as beetroot juice, was well tolerated by the subjects.

Consumption of this relatively low dose of dietary nitrate caused significant decreases in SBP and DBP (P<0.001 versus placebo control, Figure 5A and 5B). The greatest fall in SBP occurred between 3- and 6-hour post dietary nitrate ingestion with a peak mean fall of 11.2±2.6 mm Hg versus 0.7±1.9 mm Hg in the control limb. At 24 hours, clinic SBP was still significantly lower than the control limb (difference between nitrate versus water, 8.5±1.3 mm Hg; P<0.01) and reduced from baseline (7.2±2.1 mm Hg; P<0.05; Figure 5A). DBP remained lower in the dietary nitrate-treated limb up to 6 hours, with a peak mean fall of 9.6±1.2 mm Hg and returned

Table 2. Hemodynamic/Biochemical Parameters of Volunteers at Screening

Parameter	
Subjects, n	15
ABP (daytime mean), mm Hg	
Systolic	151.5±1.8
Diastolic	89.7±2.2
eGFR (MDRD), mL/min	77.3±3.3
total serum [cholesterol] (fasting), mmol/L	5.7±0.3
serum [LDL cholesterol] (fasting), mmol/L	3.3±0.3
plasma [glucose] (fasting), mmol/L	5.5±0.3

Data are shown as mean±SEM values. ABP measurements were taken with a Spacelabs 90207 (Spacelabs Healthcare, Issaquah, WA). ABP values adjusted as per British Hypertension Society guidelines (Williams et al[29]).

ABP indicates ambulatory blood pressure; eGFR, estimated glomerular filtration rate; LDL, low-density lipoprotein; and MDRD, modification of diet in renal disease formula.

to the pretreatment pressure after 24 hours (Figure 5B). No significant differences in heart rate between the 2 groups were observed (Figure 5C).

We also assessed vascular compliance using PWV at 3 hours after intervention, a timepoint that coincides with peak nitrite levels and BP-lowering.[26] PWV was significantly decreased after dietary nitrate ingestion with no change in the control limb and no differences between the 2 groups at baseline (Figure 5D).

Hypotensive Effect of Dietary Nitrate Parallels Rise in Circulating Nitrite Levels in Patients With Hypertension

After dietary nitrate ingestion, plasma [nitrate] increased rapidly above baseline with significant elevations evident at 30 minutes. Levels peaked at 2 hours (peak increase from baseline, $156.8±22.9×10^{-6}$ mol/L; $P<0.001$) and remained elevated for the first 6 hours of measurement, returning to baseline at 24 hours (Figure 5E). Plasma [nitrite] had a slower

Table 3. Demographic Characteristics and Baseline (Pretreatment) Hemodynamic/Biochemical Parameters

Characteristic	Placebo Limb	Dietary NO_3^- Limb	Significance
Subjects, n		15	
Age, y		52.9±3.7	
BMI, kg/m²		26.2±0.9	
Serum [creatinine] ($×10^{-6}$ mol/L)		89.4±3.8	
Baseline clinic SBP, mm Hg	140.2±3.6	139.9±3.9	$P=0.90$
Baseline clinic DBP, mm Hg	86.2±2.7	86.5±2.6	$P=0.80$
Plasma [NO_3^-] ($×10^{-6}$ mol/L)	33.7±4.6	46.8±10.3	$P=0.15$
Plasma [NO_2^-] ($×10^{-6}$ mol/L)	0.41±0.05	0.43±0.04	$P=0.68$

Data are shown as mean±SEM values. Significance values for paired Student t test shown in last column. Baseline blood pressure and heart rate measurements were taken in triplicate in the seated position using an Omron 715IT (Omron Corporation, Tokyo, Japan). Measurements were made every 15 min for 1 h to provide a final baseline value.

BMI indicates body mass index; DBP, diastolic blood pressure; NO_3^-, nitrate; NO_2^-, nitrite; and SBP, systolic blood pressure.

rise, becoming significantly elevated compared with placebo control at 4 hours (difference $0.24±0.06×10^{-6}$ mol/L; $P<0.05$; Figure 5F). In contrast, there were no changes in plasma [nitrite] or [nitrate] at any timepoint in the control limb (Figure 5E and 5F).

To determine whether the dietary intervention affected the level of key electrolytes that might impact on BP, we measured the serum levels of potassium (K^+), chloride (Cl^-), bicarbonate (HCO_3^-), urea, and creatinine. In these volunteers, serum [K^+] decreased after either placebo or dietary nitrate ingestion, but the decrease was greater in the placebo limb (Figure S3A). However, despite these changes, there was no correlation between [K^+] and ΔSBP observed ($P=0.396$; Figure S3B). No significant differences in serum levels were observed between the 2 limbs for [HCO_3^-], [urea], or [creatinine] (Figure S2C–S2E). Serum [Cl^-] was higher after dietary nitrate consumption but not elevated compared with baseline (Figure S3F).

Dietary Nitrate-Induced Changes in BP Are Correlated With Plasma [Nitrite]

Figure 6A demonstrates a significant correlation between change in SBP with change in plasma [nitrite] ($P<0.001$; $r=-0.280$), but not plasma [nitrate] ($P=0.120$; Figure 6B). In addition, as expected the peak decrease in SBP was correlated to baseline SBP ($P<0.05$; $r=-0.561$; Figure 6C), although not for DBP ($P=0.890$). Finally, the peak decrease in BP also correlated to baseline plasma [nitrite] ($P<0.05$; $r=-0.376$; Figure 6D).

Is the Erythrocyte the Site for Nitrite Reduction to NO in Hypertensive Patients?

To explore whether erythrocyte XOR might act as a nitrite reductase in patients, we isolated erythrocytes from the blood of 11 grade 1 hypertensive volunteers before any intervention and assessed nitrite reductase activity. Figure 7 demonstrates that nitrite causes concentration-dependent NO generation significantly attenuated by allopurinol (Figure 7B). Moreover, this raised activity was associated with higher levels of XOR expression of erythrocytes in comparison to the levels of expression observed in erythrocytes of normotensives volunteers (Figure 7C and Figure S1B). Finally, to confirm that the effects of nitrite in the patients related to NO bioactivity, we measured the circulating levels of cGMP. At 24 hours, postdietary nitrate (a time point at which BP had been sustained at lower levels for several hours) [cGMP] was significantly elevated above placebo control (Figure 7D; $P<0.05$).

Discussion

Herein, we demonstrate that the BP-lowering effects of nitrite are enhanced in an experimental model of hypertension and that these effects of nitrite, at least in part, are because of the nitrite reductase activity of erythrocytic XOR. We translate these observations to the clinical setting by demonstration in a proof-of-principle study in hypertensive patients, of similar enhanced bioactivity of nitrite, achieved through dietary provision of inorganic nitrate, and involvement of erythrocytic XOR.
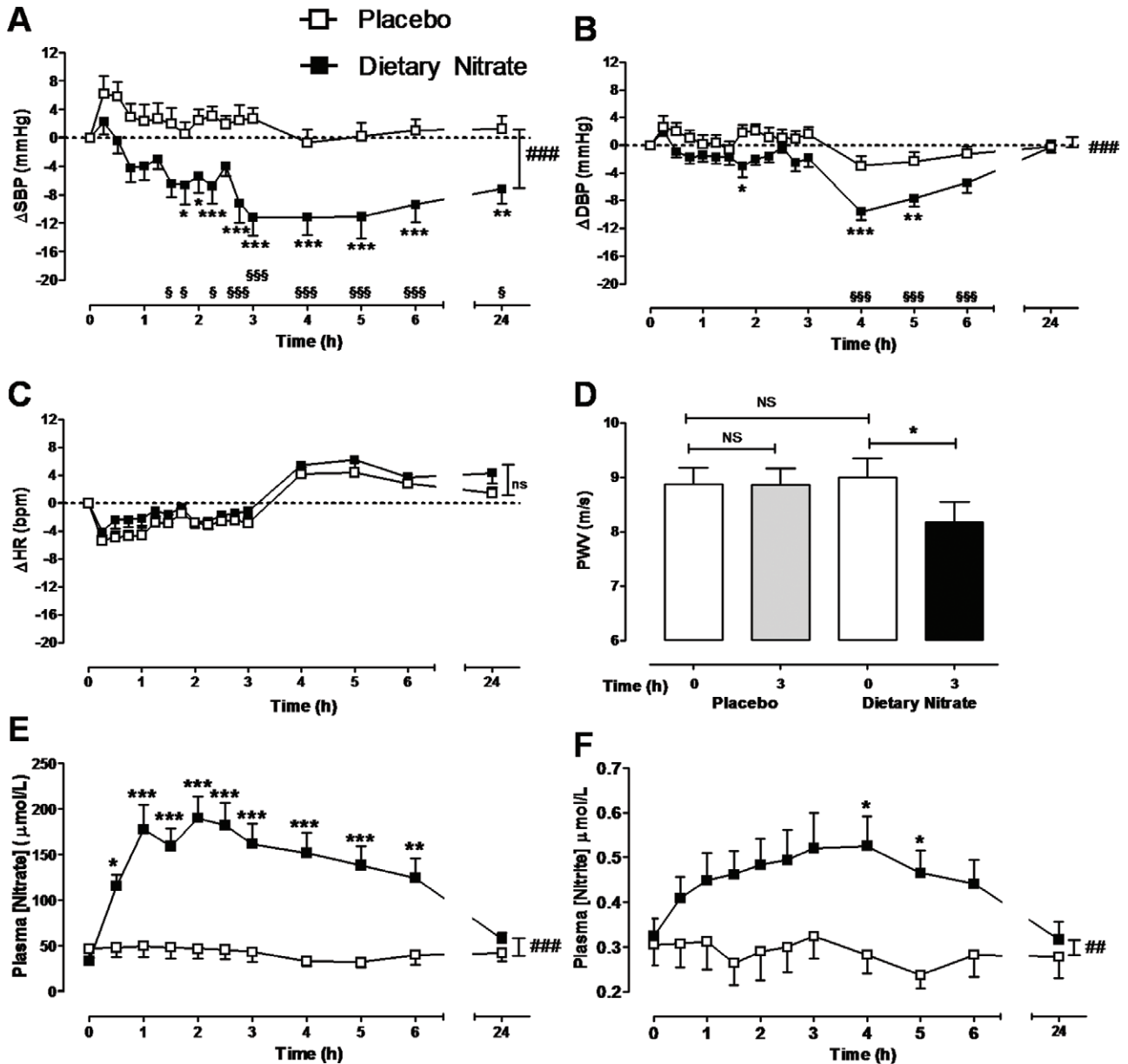
Figure 5. Dietary nitrate supplementation reduces blood pressure and elevates circulating nitrate and nitrite in grade 1 hypertensives. The effects of dietary nitrate ingestion (beetroot juice, 250 mL) or placebo (water, 250 mL) on (**A**) SBP, (**B**) DBP, (**C**) HR, (**D**) PWV, (**E**) circulating plasma (nitrate), and (**F**) (nitrite). Data expressed as mean±SEM of n=15. Significance shown for comparisons between groups as ##$P<0.01$ and ###$P<0.001$ for 2-way ANOVA; *$P<0.05$, **$P<0.01$, and ***$P<0.001$ for Bonferroni post hoc tests; and §$P<0.05$ and §§§$P<0.001$ for Dunnett post hoc tests for comparison to baseline (t =0 h) following 1-way ANOVA, after nitrate ingestion. DBP indicates diastolic blood pressure; HR, heart rate; PWV, pulse wave velocity; and SBP, systolic blood pressure.

Nitrite caused dose-dependent decreases in BP in SHR, with significant decreases becoming first evident at circulating concentrations of nitrite of ≈$1×10^{-6}$ mol/L. This concentration falls within the range of physiological levels of circulating nitrite, that is, 0.2 to $5.0×10^{-6}$ mol/L that have been reported in healthy humans.[26,30–32] Crucially at this dose, nitrite had minimal effects on BP in the normotensive WKY control. In our studies, the potassium nitrite salt decreased BP in a dose-dependent manner, in SHRs, up to the highest dose tested of $3×10^{-5}$ mol/kg, which would give an estimated circulating concentration of $1×10^{-4}$ mol/L. Importantly, although this dose of nitrite salt caused a decrease in BP of ≈32 mmHg

in SHR animals, an equivalent dose of a potassium chloride salt caused only a 5 mmHg decrease, demonstrating that the effects of the nitrite were not related to the potassium cation. We used the potassium salt of nitrite in our studies, as opposed to the often used sodium nitrite salt, because this salt is a natural corollary to dietary interventions that elevate circulating nitrite levels. As mentioned, dietary nitrate is a major source of endogenous nitrite levels. This nitrate is found in substantial amounts in vegetables, such as beetroot, lettuce, and other green leafy vegetables, that contain little sodium but significant potassium,[26] and this fact provides the rationale for use of potassium nitrite in these studies.
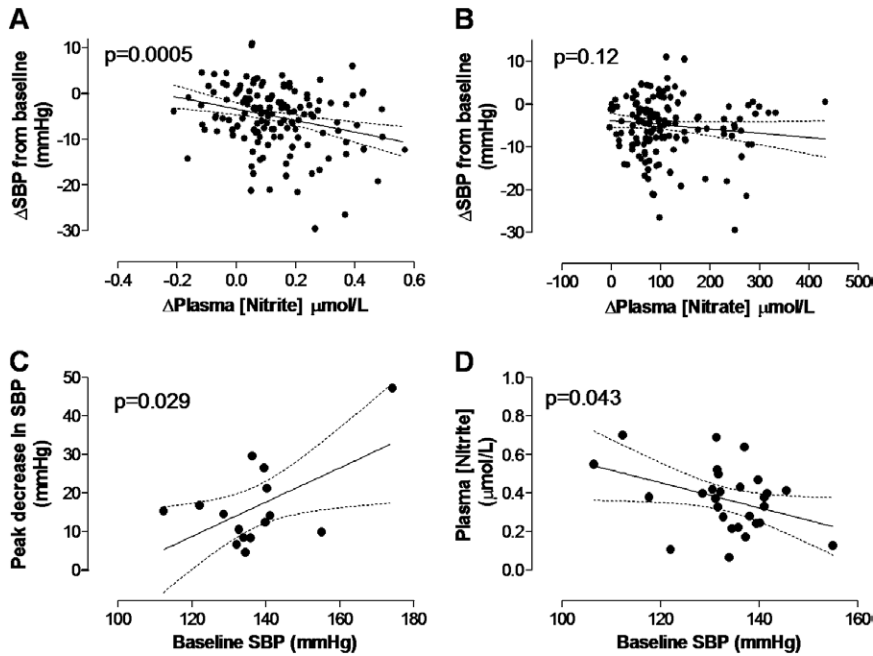
Figure 6. Plasma [Nitrite] determines BP. Correlation of changes in (**A**) [nitrite] and (**B**) [nitrate] to changes in SBP after dietary nitrate ingestion; (**C**) peak changes in SBP to baseline BP, and (**D**) correlation of baseline SBP to baseline [nitrite]. All graphs show Pearson linear regression of best-fit±95% confidence intervals. BP indicates blood pressure; and SBP, systolic blood pressure.

The hypotensive effect of the NO donor SNP was also enhanced in SHRs. A possible explanation for this difference in SNP responses is sensitization of downstream pathways in the classical NO-signaling paradigm as has been proposed to occur in eNOS-deficient mice.[33] However, the more likely explanation relates to the increased baseline BP in SHR compared with WKY. It is well-accepted that the effect of BP-lowering drugs in patients is proportional to pretreatment BP[34], that is, the higher the resting BP the greater the BP-lowering effects of vasodilator drugs. Such observations intimate that in an environment of increased vascular tone, the vasculature becomes acutely sensitive to vasodilator effects. Accordingly, raising the BP of WKY rats, to a level

equivalent to that in SHRs, resulted in an increased activity of SNP. However, in contrast the responses to nitrite were not improved intimating that the enhanced nitrite bioactivity in SHR was not simply because of enhanced baseline tone and BP. We hypothesized, therefore, that the enhanced nitrite bioactivity in SHR might reflect an upregulation of the pathways for nitrite bioactivation.

It has been suggested that the predominant nitrite reductase activity within the circulation is localized to the blood vessel wall and that a major component of this activity is provided by XOR.[24] Enzyme studies with purified XOR have shown that in addition to its conventional role in purine catabolism, it also reduces nitrite to NO.[35,36] Importantly, XOR activity
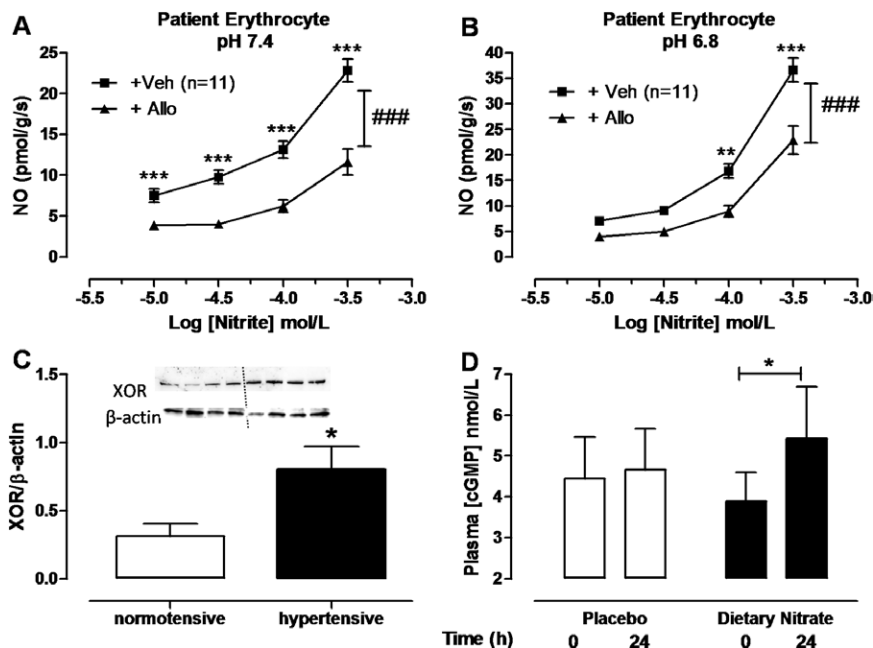

Figure 7. Xanthine oxidoreductase (XOR) facilitates nitrite-derived NO generation by erythrocytes of hypertensives. Comparison of the nitrite reductase activity of purified erythrocytes at pH 7.4 (**A**) and pH 6.8 (**B**) and in the presence of vehicle (veh) or the XOR inhibitor allopurinol (Allo, 100 μmol/L), and (**C**) the expression of XOR of erythrocytes purified from the blood of normotensives and hypertensive volunteers (n=4 for each) determined by Western blotting and, (**D**) the effects of dietary nitrate ingestion (beetroot juice, 250 mL) or placebo (water, 250 mL) on plasma (cyclic GMP) (n=15). Data expressed as mean±SEM. Significance shown for comparisons between curves as #$P<0.05$ and ###$P<0.001$ using repeated measures of 2-way ANOVA followed by Bonferroni post tests shown as **$P<0.01$ and ***$P<0.001$. Unpaired Student t tests for XOR expression and repeated measures of 1-way ANOVA with Bonferroni post tests for cGMP levels respectively with statistical significance shown as *$P<0.05$.

and expression are increased in models of hypertension, including the SHR,[37,38] and thought to be pathogenic; effects linked to XOR-mediated superoxide generation occurring at the flavin adenine nucleotide (FAD) site of the enzyme.[39] Indeed, recent evidence suggests that inhibition of XOR activity, using allopurinol, in patients with stable angina or heart failure[40–43] improves several cardiovascular outcome indices, including severity of disease as well as vascular function. Interestingly, it has also been demonstrated that in individuals with few hypertensive years allopurinol treatment lowers BP, an effect that is lost in individuals suffering from hypertension for prolonged periods of time.[44,45] It is postulated that the beneficial effects of allopurinol relate, at least in part, to block of the superoxide generating activity of the FAD site of XOR and thus reduced oxidative stress. However, it is the molybdenum-containing subunit of XOR that has been identified as a key site for nitrite reductase activity in models of cardiovascular disease.[46,47] Thus, we have previously speculated that, at XOR, nitrite might compete with oxygen for electrons. Moreover, that providing XOR with nitrite as an alternative substrate may result in functional effects due not only to the generation of NO but also to inhibition of reactive oxygen species production.[48] In accord with this accumulating evidence, treatment of SHR with allopurinol profoundly suppressed the BP-lowering effects of nitrite.

To test the hypothesis that blood vessel wall–associated XOR might underpin the enhanced hypotensive activity of nitrite in SHR, we isolated conduit and resistance arteries to assess the vasorelaxant activity of nitrite in vitro. However, in contrast to our in vivo observations, nitrite-induced vasorelaxation was decreased in SHR compared with WKY. A similar depression of reactivity was evident in response to the NO donor spermine-NO, suggesting reduced sensitivity of the NO-soluble guanylate cyclase pathway or alternatively enhanced metabolism by phosphodiesterase in hypertension. Indeed, with respect to the latter possibility, blockade of phosphodiesterase 5 activity decreases BP in mild to moderate hypertension in patients.[49] Importantly, inhibition of XOR and a range of other putative nitrite reductases did not attenuate nitrite bioactivity in either strain, although L-NMMA or endothelial denudation did enhance responses in arteries of SHR. A possible explanation for this latter effect is that uncoupling of eNOS in hypertension results in diminished NO generation with a switch to superoxide generation by the enzyme.[50] Inhibition of eNOS might, therefore, decrease superoxide levels and in so doing increase the effect of nitrite by enhancing the bioavailability of the NO generated. It is also noteworthy that concentrations of 1 to 10×10^{-5} mol/L nitrite were required before relaxation was evident, whereas in vivo concentrations of 1 to 3×10^{-6} mol/L were sufficient to see significant decreases in BP. Together, these observations suggest that the chemical reduction of nitrite at the blood vessel wall does not account for the effects of nitrite on BP in vivo.

Because we saw no effect of allopurinol at the level of the blood vessel wall, we investigated the possibility that the effects of XOR inhibition in vivo might relate to an effect at the level of another proposed important site for nitrite reduction, the erythrocyte.[15] Indeed,

allopurinol virtually abolished the nitrite reductase activity of erythrocytes purified from SHR, but not WKY animals. Moreover, erythrocytic nitrite reductase activity was substantially enhanced in the SHR versus the WKY, a pattern of activity that fits well with the in vivo data. This enhancement of activity may be attributed to an increase in the levels of erythrocytic XOR expression, as demonstrated by a doubling of levels measured by Western blotting. Indeed, in line with previous studies, we identified increased XOR expression in the liver of SHR[37,38] and the erythrocyte, but not in the blood vessels. The liver is one of the major sites for XOR synthesis releasing XOR into the circulation,[51] especially after periods of metabolic stress,[52] which is then carried to distant parts of the circulatory tree where it binds to glycosaminoglycans found on the surfaces of circulating cells, including erythrocytes, and on the endothelium.

Importantly, allopurinol also significantly attenuated the nitrite reductase activity of erythrocytes of hypertensive patients. This observation contrasts, but dovetails well, with our own previous investigations in normotensives where erythrocytic nitrite reduction was unaffected by XOR inhibition at physiological pH.[53] These observations are in accord with work demonstrating no effect of allopurinol on nitrite-induced vasodilatation in the forearm of healthy volunteers.[14] Thus, although XOR does not seem to contribute to nitrite reduction in health, it does seem to mediate nitrite reduction in pathological scenarios.

We also show that erythrocytic XOR expression is doubled in hypertensives in comparison with normotensives, a picture that closely resembles our findings in the rat model of hypertension. Importantly, erythrocytic nitrite and nitrate levels were found to be significantly lower in SHR compared with WKY (Figure 4G and 4H). An explanation for this phenomenon is that it reflects an increased consumption of nitrite basally in the SHR attributed to an upregulation of nitrite reductase pathways, a phenomenon proposed to underlie the arteriovenous gradient in erythrocyte nitrite concentrations in humans.[54,55] Together, our findings suggest a hypertension-specific elevation of erythrocytic XOR-dependent nitrite reductase capacity that likely underlies the BP-lowering effects of nitrite in rats.

That nitrite might similarly be more potent in hypertensive patients is supported by our proof-of-principle studies demonstrating that elevation of circulating nitrite, to levels previously shown to be insufficient to cause significant BP-lowering in healthy volunteers, caused substantial and significant decreases in BP. We used dietary nitrate administration as a method to elevate circulating nitrite levels and found that, similar to healthy volunteers,[25,26] after ingestion there is a slow-developing rise in plasma [nitrite], with the peak effect occurring at ≈4 hours in patients. This time-lag reflects the dependence on the enterosalivary circuit for the reduction of nitrate to nitrite in the body.[7] Participants received a dose of ≈3.5×10^{-3} mol nitrate that caused a 1.5±0.1-fold increase of plasma [nitrite]. This rise is similar to the 1.4-fold rise in circulating nitrite achieved by a similar dose of 4×10^{-3} mol inorganic nitrate in healthy volunteers and indicates that hypertension per se does not alter the

bioconversion rate and extent of nitrate processing via the enterosalivary circuit.

As with the studies in animals, we demonstrate an increased potency of nitrite in patients. The peak BP drop after dietary nitrate ingestion in this study was 11.2/9.6 mm Hg. Equivalent doses in our previous healthy volunteer study produced modest decreases in BP of 1.7/4.6 mm Hg.[25] This enhanced sensitivity could simply reflect the higher baseline BP evident in the hypertensive cohort, although our experiments in normotensive rats with artificially raised BP demonstrating no enhancement of nitrite bioactivity would argue against this being the sole determinant. Our findings suggest that this enhanced bioactivity is probably a reflection of the hypertension-specific enhanced nitrite reductase activity of erythrocytes, mediated by XOR, resulting in NO-mediated vasodilatation. This role of NO is supported by demonstration of elevations in plasma cGMP levels together with observations that PWV is decreased. It is possible that PWV decrease is simply secondary to the decrease in BP, although other studies have shown acute reductions in PWV (within 4 hours), after the administration of vasoactive compounds, more than would be expected consequent to BP-lowering on its own.[56] Interestingly, the BP-lowering effects persisted at the 24-hour time point when circulating levels of nitrate and nitrite had returned almost to baseline. It has been demonstrated that nitrite within the circulation is rapidly taken up into tissues[57] where it may be stored. It is possible that, despite diminution of circulating nitrite levels, tissue levels remain elevated providing a continued supply of NO, although further mechanistic studies exploring this possibility are warranted.

Limitations

In the patient proof-of-principle study, we assessed the effects of a single dose of dietary nitrate, in the form of beetroot juice, over a 24-hour period and used an equal volume of low NOx-containing water as a control. It is possible that the open-label nature of the design may have impacted on the BP effects evidenced. More recently, a nitrate-free juice placebo has been developed[58] and future studies incorporating such a placebo would be advantageous to exclude any possibility that the effects seen are attributed to an effect independent of the nitrate content. It is noteworthy, however, that the effects of dietary nitrate have been replicated in double-blind studies using equivalent doses of KNO_3 versus KCl placebo.[25,59] Moreover, the effects of dietary nitrate administration in the form of beetroot juice have been shown unequivocally to be dependent on the enterosalivary bioactivation to nitrite. In addition, the BP-lowering effects of the dietary intervention are directly correlated to the rise in circulating nitrite levels.[26] A second limitation of our study is the short-term nature of BP-lowering effects. Whether long-term elevation of dietary nitrate intake can provide sustained elevations in nitrite and therefore decreases in BP is unknown. Such investigations are essential to determine the true potential use of such an intervention in hypertensive individuals. Finally, although our study was adequately powered, for any therapy to become part of the established armamentarium large-scale outcome trials are needed.

Perspectives

In summary, we have demonstrated that nitrite is more potent at reducing BP in hypertensive animals and patients than in normotensives. Moreover, we show that this increased bioactivity is related not only to the raised resting BP but also to an increased activity of XOR. Perhaps more importantly, we show that a dietary nitrate load (in the form of beetroot juice), at a dose that is easily achievable, sufficiently elevates circulating plasma nitrite levels with resulting reductions in BP. Given that ≈50% of treated hypertensive subjects fail to achieve their target BP,[4] an additional strategy, based on intake of nitrate-rich vegetables, may prove to be cost-effective, easily achievable, safe, and favorable for public health. Our proof-of-principle study highlights the imperative to ascertain this potential in patients.

Disclosures

A. Ahluwalia and N. Benjamin are directors of Heartbeet Ltd.

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Novelty and Significance

What Is New?

- Dietary nitrate lowers blood pressure in patients with hypertension.
- Inorganic nitrate/nitrite is more potent in hypertensives than normotensives, the blood pressure–lowering effects are attributable to the conversion of nitrate to nitric oxide in vivo.
- Increased xanthine oxidoreductase localized to the erythrocyte in hypertension plays an important role in the generation of nitric oxide from ingested inorganic nitrate.

What Is Relevant?

- Oral ingestion of a single dose of dietary nitrate exerts potent and long-lasting blood pressure decrease in hypertension.

Summary

Systemic nitrite elevation may prove useful in the therapeutics of hypertension.