Dietary nitrate ameliorates pulmonary hypertension: cytoprotective role for endothelial nitric oxide synthase and xanthine oxidoreductase

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Abstract

Background—Pulmonary hypertension (PH) is a multi-factorial disease characterized by increased pulmonary vascular resistance and right ventricular failure; morbidity and mortality remain unacceptably high. Loss of nitric oxide (NO) bioactivity is thought to contribute to the pathogenesis of PH and agents that augment pulmonary NO signaling are clinically effective in the disease. Inorganic nitrate (NO_3^-) and nitrite (NO_2^-) elicit a reduction in systemic blood pressure in healthy individuals; this effect is underpinned by endogenous and sequential reduction to NO. Herein, we determined whether dietary nitrate and nitrite might be preferentially reduced to NO by the hypoxia associated with PH, and thereby offer a convenient, inexpensive method of supplementing NO functionality to reduce disease severity.

Methods & Results—Dietary nitrate reduced the right ventricular pressure and hypertrophy, and pulmonary vascular re-modeling, in wild-type mice exposed to 3 weeks hypoxia; this beneficial activity was mirrored largely by dietary nitrite. The cytoprotective effects of dietary nitrate were associated with increased plasma & lung concentrations of nitrite and cGMP. The beneficial effects of dietary nitrate and nitrite were reduced in mice lacking endothelial NO synthase (eNOS) or treated with the xanthine oxidoreductase (XOR) inhibitor allopurinol.

Conclusions—These data demonstrate that dietary nitrate, and to a lesser extent dietary nitrite, elicit pulmonary dilatation, prevent pulmonary vascular remodeling, and reduce the RVH characteristic of PH. This favorable pharmacodynamic profile is dependent on eNOS and XOR - catalyzed reduction of nitrite to NO. Exploitation of this mechanism (i.e. dietary nitrate/nitrite supplementation) represents a viable, orally-active therapy for PH.

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Keywords

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INTRODUCTION

Pulmonary hypertension (PH) encompasses numerous etiologically distinct pathologies that manifest as increased pulmonary arterial blood pressure, vascular remodeling of the pulmonary resistance vasculature, right ventricular hypertrophy (RVH) and ultimately right ventricular failure[1]; 2-year mortality remains high at ~15%[2]. The disease is progressive and current therapy, including prostacyclin analogues[3], endothelin receptor antagonists[4] and phosphodiesterase 5 inhibitors (PDE5i)[5] slow, but do not halt, pathological deterioration. The condition therefore represents a clear unmet medical need, and this therapeutic deficit is likely to widen in the future with the increasing incidence of precipitating conditions such as chronic obstructive pulmonary disease (COPD) and interstitial lung disease[6, 7].

Loss of nitric oxide (NO) bioactivity, leading to deficient soluble guanylate cyclase (sGC) activation and cyclic guanosine-3′,5′-monophosphate (cGMP) production, underpins many of the hemodynamic and morphological changes in the cardiopulmonary circulation that characterize PH[8], particularly pulmonary arterial hypertension. Accordingly, therapeutic approaches that supplement NO-dependent signaling, including NO inhalation[9], sGC activators[10] and PDE5i[5], are clinically effective in patients with the disease. Furthermore, therapeutic combinations targeting lung cGMP (e.g. inhaled NO & PDE5i[11], inhaled NO & sGC activators[12], natriuretic peptides & PDE5i[13]) produce an additive or synergistic benefit in alleviating pathology. This approach is effective, at least in part, because elevating cGMP concentrations reverses both the hemodynamic and structural changes in the lung[14, 15], a capacity that is perceived to be a prerequisite for advancing therapy.

Recently, it has emerged that the NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-) can be chemically reduced *in vivo* to biologically active NO; a phenomenon that occurs optimally under conditions of hypoxia and acidosis[16]. This novel means of generating cytoprotective NO appears to be dependent on reduction of nitrate to nitrite by facultative anaerobes on the dorsal surface of the tongue, entry of the nitrite into the entero-salivary circulation, transit to the stomach and absorption through the gut wall[17]. Conversion of nitrite to NO is then facilitated by a family of (hemo)proteins that exhibit 'nitrite reductase' activity, including xanthine oxidoreductase (XOR)[18-20], globins[21-24], aldehyde oxidase[25], and even endothelial NO synthase (eNOS)[26, 27]. This nitrate-nitrite-NO pathway has been shown to exert a number of beneficial effects including lowering of systemic blood pressure and protection against ischemia-reperfusion (I/R) injury[20, 24, 28, 29]. Indeed, ingestion of (inorganic) nitrate may underlie the cardioprotective phenotype of a diet rich in fruits & vegetables[30, 31].

In the context of the pulmonary vasculature, inhaled or infused nitrite has been shown to be effective in producing acute pulmonary vasodilatation and to reduce severity in models of PH[32-34]. However, the long-term treatment of patients with PH would likely be better accomplished by an orally-active supplementation (either dietary or pharmacological) of NO bioactivity, particularly considering the short plasma half-life of inhaled or intravenous nitrite (<1hr)[34-36] and the potential for nitrite-induced toxicity (e.g. methemoglobinemia[36]).

Therefore, in the present study we have investigated the hypothesis that dietary nitrate, via sustained sub-micromolar elevations in circulating nitrite concentrations, prevents the development of hypoxia and bleomycin -induced PH. In addition, we have probed the

'nitrite reductase' mechanism of effects observed using eNOS deficient mice and the XOR inhibitor allopurinol.

METHODS

Hypoxia-induced PH

All studies conformed to the UK Animals (Scientific Procedures) Act 1986. Wild-type (WT) or eNOS knockout (KO) littermates (male, 20-25g; C57BLK6 background) were randomly assigned to one of 5 groups: [1] normoxia, [2] hypoxia controls (10% O_2; normobaric; 3 weeks), [3] hypoxia with nitrite (0.6mM), [4] hypoxia with nitrate (15mM), [5] hypoxia with nitrate (45mM; all interventions were administered in the drinking water). In additional studies, mice were treated with the XOR inhibitor allopurinol (1mM in drinking water; dose shown previously to prevent XOR activity *in vivo*[37]) in the absence and presence of nitrate (45mM). Treatment was initiated 2 days prior to hypoxia. The final doses of nitrite and nitrate were calculated by weighing drinking bottles daily to determine the volume of water consumed (Table 1). Potassium nitrite and potassium nitrate were purchased from Sigma Chemical Co (Dorset, U.K.). Drinking water solutions were made freshly and replaced every 2 days. Untreated water contained <50nM nitrite and nitrate (Table 1). In representative experiments, urine output was collected from specially adapted metabolic cages that contained two mice within the same treatment group. Studies were also conducted in which dietary NO_3^- (45mM) was introduced after 2 weeks of hypoxia (i.e. following onset of overt PH) and hemodynamic measurements made following 3 further weeks exposure to hypoxia (i.e. to assess the potential of dietary nitrate to reverse established pathology).

Bleomycin-induced PH

A second, etiologically-distinct model of PH was employed to validate the efficacy of dietary nitrate in reducing disease severity[38]. Mice (male; 20-25g; C57BLK6) were exposed to bleomycin (30μl/mouse; 1mg/kg) by oropharangeal instillation under light isofluorane induced anesthesia. Controls were similarly instilled with 30 μl of sterile saline. Animals were randomly assigned to one of 3 groups: [1] Control, [2] Bleomycin-treated [3] Bleomycin with nitrate (45mM; administered in the drinking water). Treatment was started concomitantly with the administration of bleomycin (i.e. day 1) and hemodynamics assessed at day 14.

Hemodynamic measurements

The right ventricular systolic pressure (RVSP), mean arterial blood pressure (MABP) and RVH (as calculated by right ventricle to left ventricle plus septum ratio; RV/LV+S) were determined, as we have described previously[13]. The left lung was then fixed by inflation with 10% formalin in PBS before paraffin embedding and sectioning. The remaining lung tissue, heart and kidney were dissected and snap frozen in liquid N_2. Plasma was collected by centrifugation (220 × g; 20 min, 4°C) of whole blood and stored at −80°C. In representative experiments, urine was collected in a specialized metabolic chamber housing two animals (receiving the same intervention) concomitantly. Samples were then assayed for nitrite and nitrate (by chemiluminescence) and/or cGMP content (cGMP EIA Biotrak System, GE Healthcare UK Ltd) as we have previously described[13].

Lung nitrite reductase activity

NO production from homogenized lung supernatants from mice exposed to 3 weeks hypoxia in the presence of sodium nitrite (10 to 300μM; pH 5.5) was determined as we have described previously[20]. To investigate the role of eNOS and XOR in tissue-dependent nitrite reduction samples were incubated with the NOS inhibitor N[G]-methyl-L-arginine (L-NMA;

300 μM) or the XOR inhibitor allopurinol (100 μM), respectively. All drug pretreatments were for 30 minutes before nitrite incubation.

Morphological analysis

Transverse formalin-fixed lung sections were stained with an anti-α-smooth muscle actin (clone 1A4; Dako, Ely, UK) antibody. Pulmonary arterial muscularization was then assessed as previously described[39]. Briefly, vessels were grouped according to diameter (<500μm, 500-1000μm, >1000μm) and defined according to presence or absence of muscularization. Twenty five muscularized arteries from different fields were then imaged at 400× magnification by light microscopy from representative animals in each group to determine wall diameter.

Data analysis

Data were analyzed by one way ANOVA followed by a Bonferroni post-test, with the exception of (a) nitrite reductase activity which was analyzed across the entire concentration range by two way ANOVA, and (b) the relationship between lung [nitrite] and plasma [cGMP] which was established by resolving the correlation coefficient (i.e. coefficient of determination, R^2). Results are expressed as mean ± s.e.mean, and *P*<0.05 denotes significance. The *n* value denotes the number of animals used in each group.

RESULTS

Effect of oral nitrite and nitrate supplementation on right ventricular pressure

In untreated control mice, 3 weeks of 10% hypoxia produced markedly elevated RVSP compared to normoxia controls (Figure 1). Animals treated with nitrite (0.6mM) and the higher dose of nitrate (45mM) showed a statistically significant reduction in RVSP compared to untreated hypoxic animals (Figure 1). Treatment with nitrate (45mM) virtually abolished the rise in RVSP in response to hypoxia. The lower dose of nitrate (15mM) trended towards improving RVSP and provided evidence for a dose-dependent effect of dietary nitrate.

Effect of oral nitrite and nitrate on RVH

There were no significant changes in total ventricle weight across the groups (data not shown). Exposure to hypoxia resulted in a significant increase in RV/LV+S ratio, indicative of the well-characterized RVH that occurs in PH (Figure 1). Akin to observations made with respect to RVSP, treatment with nitrite (0.5mM) or the higher dose of nitrate (45mM) significantly reduced the hypoxia-induced increase in RV mass. However, treatment with the lower dose (15mM) of nitrate did not prevent the RVH associated with hypoxia (Figure 1).

Effect oral nitrite and nitrate on pulmonary vascular remodeling

Normoxic animals showed only a modest degree of pulmonary muscularization (Figure 2) with typical wall thicknesses of <4μm (Figure 3). Exposure to 3 weeks of hypoxia resulted in a dramatic increase in the number of muscularized pulmonary arteries; this change was true of all branches of the pulmonary vascular tree (Figure 3). Treatment with nitrite (0.6mM) caused a small reduction in the proportion of muscularized vessels (Figure 2), but did not significantly reduce mean wall thickness (Figure 3). In contrast, nitrate elicited a significant, dose-dependent reduction in the percentage of vessels that had become muscularized (Figure 2), and the mean wall thickness (Figure 3). This beneficial effect of dietary nitrate against pulmonary vascular remodeling was apparent in small (<500μm), medium (500-1000μm) and large (>1000μm) pulmonary vessels (Figure 3), suggesting

nitrate therapy is efficacious in reducing remodeling of the resistance arteries, which predominantly determine pulmonary pressure & resistance.

Effect of oral nitrite and nitrate on plasma [nitrite], [nitrate] and [cGMP]

Ingestion of nitrite (0.6mM) and nitrate (15mM & 45mM) significantly increased plasma concentrations of nitrite under normoxic conditions. Furthermore, ingestion of nitrate (15mM & 45mM) elevated plasma nitrate concentrations under the same conditions (Figure 4). A very different pattern was observed under hypoxia. At this lower pO_2, nitrite administration in the drinking water did not alter plasma nitrite or nitrate concentrations (Figure 4). Yet, administration of the higher dose of nitrate (45mM) under such circumstances still resulted in a significant increase in plasma nitrite concentrations. Akin to the picture in normoxic control mice, ingestion of nitrate caused a significant rise in plasma nitrate concentrations under hypoxic conditions (Figure 4).

Dietary administration of nitrite and nitrate failed to increase plasma cGMP concentrations under normoxia. However, in mice exposed to 3 weeks hypoxia, oral nitrite and nitrate therapy tended to increase plasma cGMP concentrations, although statistical significance was only achieved with 45mM nitrate (Figure 4).

Effect of oral nitrite and nitrate on tissue & urine [nitrite] and [nitrate]

Tissue—Total lung concentrations of nitrite were not significantly different for any treatment group under normoxia or hypoxia (Figure 5). However, administration of nitrate (45mM) caused a significant increase in both lung nitrite and nitrate concentrations, which in the case of tissue nitrate was substantially greater under hypoxia (Figure 5). An essentially identical pattern was observed in the heart and kidney (data not shown).

Urine—Excretion of nitrite by the kidney was increased following administration of nitrate (45mM) to normoxic animals (Figure 5). Yet, under hypoxic conditions the same concentration of nitrate did not significantly increase urinary nitrite excretion (Figure 5). These findings imply that plasma nitrite is being retained and/or utilized in a hypoxic environment. Administration of nitrate (15mM & 45mM) also caused an increase in the excreted concentration of nitrate, which were similar under normoxic and hypoxic conditions. In addition, ingestion of nitrite did not alter urinary nitrite or nitrate concentrations under normoxia or hypoxia (Figure 5).

Effect of oral nitrate on established hypoxia-induced PH, and on bleomycin-triggered PH

Dietary administration of nitrate (45mM) following the onset of overt hypoxia-induced PH significantly reversed the PH and RVH (Figure 6). Moreover, supplementation with oral nitrate (45mM) also inhibited the development of increased RVSP and RVH associated with bleomycin-induced pulmonary fibrosis (Figure 6).

Effect of eNOS gene deletion on the pharmacodynamic activity of nitrite and nitrate

In order to garner mechanistic insight regarding the nitrite reductase pathway(s) responsible for the endogenous generation of cytoprotective NO from dietary nitrite and nitrate, parallel experiments were performed in eNOS KO animals using dietary nitrite (0.6mM) and nitrate (45mM).

Under normoxic conditions, RVSP was elevated in eNOS KO mice compared to WT littermates, as has been previously described[8]. Following exposure to 3 weeks hypoxia, eNOS KO animals exhibited an exaggerated response (compared to WT mice) manifesting as a significantly greater increase in RVSP (Figure 7). In contrast to WT animals, the

beneficial effect of nitrite and nitrate to lower RVSP was significantly reduced in eNOS KO animals (Figure 7).

An essentially identical pattern of activity was observed in terms of RVH. Here, nitrite and nitrate prevented the increase in RV/LV+S ratio in WT animals (Figure 1) but was ineffective in eNOS KO animals (Figure 7).

The lack of effect of dietary nitrite and nitrate in eNOS KO mice was not due to an inability to increase plasma concentrations of these nitrogen oxide species. Both nitrite (0.6mM) and nitrate (45mM) caused a significant increase in plasma nitrite concentrations in eNOS KO animals (Figure 7); indeed, if anything the increase in plasma nitrite concentrations was greater than that occurring in WT animals (Figure 4). Oral nitrate also increased plasma nitrate concentrations in eNOS KO animals in a similar fashion to WT mice (Figure 7).

Effect of XOR inhibition on the pharmacodynamic activity of nitrite and nitrate

Since residual beneficial activity of dietary nitrite and nitrate on the development of hypoxia-induced PH was observed in eNOS KO animals, essentially identical experiments were conducted in mice exposed to three weeks hypoxia following treatment with the XOR inhibitor allopurinol (1mM) in the absence and presence of nitrate supplementation (45mM).

Following exposure to hypoxia, the presence of allopurinol alleviated the development of increased RVSP and RVH (Figure 8). This dovetails well with previous work concluding that superoxide production by XOR contributes to the pathogenesis of PH[40, 41]. In mice treated with allopurinol, the beneficial effect of dietary nitrate supplementation was reduced (Figure 8).

Biochemical determination of the lung nitrite reductase activity

The nitrite reductase activity of homogenized whole lung supernatants from mice exposed to 3 weeks hypoxia was determined. Nitrite (10-100μM) caused a concentration-dependent generation of NO (gas) that was inhibition by both L-NMA and allopurinol (Figure 8), confirming biochemically an important role for both eNOS and XOR in the beneficial effects of dietary nitrate supplementation in experimental PH.

Effect of oral nitrite and nitrate on systemic blood pressure

Across genotype and interventions there was no effect of dietary nitrite or nitrate on mean arterial blood pressure (MABP), with the exception of a reduction in MABP in response to the higher dose of nitrate (Table 2).

DISCUSSION

Oral administration of inorganic nitrate has recently emerged as a safe, effective approach to elevate circulating concentrations of NO within the physiological realm[28, 29, 31]. In the present study we explored whether dietary supplementation with inorganic nitrate might prevent the hemodynamic and morphological changes that characterize and are pathogenic in PH. Our data suggest that increasing oral nitrate intake prohibits, and also reverses, increases in RVSP, re-modeling of the small pulmonary arteries, and the RVH that characterize the disease. Furthermore, these beneficial effects are largely replicated by oral administration of inorganic nitrite, supporting the view that endogenous reduction of nitrate to nitrite underlies this response. This work also intimates that eNOS functions as the predominant nitrite reductase in PH that facilitates the generation of cytoprotective NO; however, we also provide evidence for an important role of xanthine oxidoreductase in pulmonary NO production from nitrite, as suggested by previous work[34]. These

observations advocate the use of dietary nitrate as a potentially efficacious treatment of PH that is amenable for translation to the clinical arena quickly, safely and inexpensively.

PH remains a progressive disease with limited treatment options and a high associated morbidity and mortality[15, 42]. NO-centric therapies are clinically effective in the disease[5, 9-11], and are a reflection of the bioactivity of its associated intracellular second messenger cGMP that reverses several disease pathologies[8]. Thus, efforts to maximize this beneficial profile are warranted. Under hypoxic environments, conventional NO production by NOS is impaired, and nitrite represents an alternate source of NO that can be utilized in a bid to maintain its cytoprotective influence in the vasculature[20, 43, 44]. This is achieved by the reduction of nitrite by several potential endogenous 'nitrite reductase' enzymes (e.g. XOR, globins, eNOS). Since chronic hypoxia and lowered arterial oxygen saturation are commonly associated with PH, it is reasonable to hypothesize that this endogenous nitrite to NO reductive pathway is likely to offset development of PH and represent a tangible means of pharmacologically augmenting the beneficial prolife of NO to treat this disease. Data presented herein support both theses.

We used a well-defined model of hypoxia-induced PH to assess the efficacy of dietary nitrate on several indices of disease progression. The doses of nitrate were chosen based upon data obtained in studies in animal models and healthy volunteers demonstrating the sustained elevation of plasma nitrite at low micromolar concentrations is associated with functional effects of NO[28, 31, 45]. Although the doses used in mice in the present study are greater than might be expected to be consumed with a diet rich in fruits and vegetables (e.g. consumption of inorganic nitrate following guidelines advocated by the Dietary Approaches to Stop Hypertension (DASH) study equates to ~1200mg/day[46]), the increase in plasma nitrite observed in mice is commensurate with the elevations seen in healthy volunteers who have consumed beetroot juice or KNO_3 (which are hemodynamically active[28, 31]). This difference between rodents and humans in terms of nitrite/nitrate handling may reflect a faster elimination of both anions in mice, resulting in comparatively lower plasma levels for any given dose consumed. It is therefore important to focus on achievable plasma nitrite levels, which correlate closely with hemodynamic activity, rather than absolute dose *per se*; as such, the plasma concentrations of nitrite and nitrate shown in this study are readily and realistically achievable in man. Our results demonstrate a clear protective effect of nitrate in hypoxia-induced PH. This is manifested as a reduction in RVSP, prevention of RVH, and inhibition of the re-modeling of the small pulmonary arteries. That these beneficial effects are due to the sequential reduction of nitrate is supported by the similar profile achieved through nitrite supplementation. However, of the two species it appears that dietary nitrate is the more effective agent in offsetting the hemodynamic and structural changes that characterize the disease. For similar increases in plasma nitrite, it is dietary nitrate (particularly at the 45mM dose) that produces a consistently efficacious activity across all parameters. This is highlighted by the remodeling of the pulmonary resistance vasculature. Whilst the dietary nitrite produced a trend towards inhibition of this parameter, this was not significantly different to hypoxic control animals. However, dietary nitrate elicited a dose-dependent reduction in muscularization that significantly improved the vascular response to hypoxia.

The value of therapy with dietary nitrate was reinforced by observations evaluating the plasma and tissue concentrations of components of the NO signaling pathway. Here, plasma nitrite concentrations were significantly elevated with nitrate (and nitrite) ingestion under normoxic and hypoxic conditions, with the exclusion of nitrite administration under hypoxia. This exception likely reflects the more sustained production of nitrite by dietary nitrate supplementation that maintains an elevated plasma nitrite concentration even when its reduction to bioactive NO is accelerated under hypoxic conditions; this may well underlie

the greater efficacy of dietary nitrate in this disease model. Indeed, the bioactivation of nitrite under hypoxic conditions is illustrated unmistakably when using plasma cGMP concentrations as an index of sGC activation. Here, the higher dose of nitrate elicited an increase in plasma cGMP (although both nitrite and the lower dose of nitrate also tended to enhance plasma cGMP concentrations) whereas the same dose was unable to produce this effect under normoxic conditions. Such observations infer a hypoxia-dependent production of NO and activation of sGC. Moreover, plasma cGMP concentrations correlated extremely well with nitrite concentrations found in the lung under a hypoxic environment (Figure 8), suggesting that the endogenous bioactivation of nitrite occurred at the site that needed it most; the pulmonary circulation.

Analysis of urinary excretion of nitrate and nitrite also highlighted an intriguing pattern of activity that suggests an endogenous mechanism for salvaging cytoprotective nitrite under conditions of hypoxia. Dietary nitrate caused a substantial increase in excretion of nitrite and nitrate in a normoxic environment. However, during hypoxia the excretion of nitrite was completely abolished (whereas urinary nitrate concentrations remained relatively unaltered). These data intimate that during hypoxia (a) nitrite is rapidly utilized for NO generation and/ or (b) there is a renal mechanism that retains nitrite for this purpose. It is interesting to note that the higher dose of nitrate (45mM) actually caused a significant increase in endogenous nitrite production in normoxia but this is rapidly and efficiently excreted to maintain plasma concentrations close to baseline. Thus it appears a tight physiological regulation of plasma nitrite, but not plasma nitrate, occurs that can be adapted to respond to pathophysiological situations (e.g. hypoxia) when nitrite reduction is beneficial; this also appears to hold true for pharmacological manipulation, particularly with dietary nitrate.

Since prophylactic treatment (as above) does not mirror the clinical scenario, in which patients present with symptomatic PH, studies were performed to evaluate the ability of dietary nitrate supplementation to reverse the elevated pulmonary artery pressure and RVH associated with established disease. Akin to the observations made in initial studies, nitrate ingestion produced a significant reversal of established PH, reducing the pulmonary hemodynamic changes and cardiac remodeling. Further studies were undertaken to demonstrate the efficacy of inorganic nitrate in an etiologically-distinct model of PH, that secondary to bleomycin-induced pulmonary fibrosis. Analogous observations were made in this setting; dietary supplementation with inorganic nitrate prevented the elevated RVSP and RVH. These data intimate that the beneficial effects of nitrate-based therapy are not model-specific and are apparent in an additional experimental system with a very different etiology. Moreover, this outcome provides optimism that dietary supplementation with nitrate has potential to be efficacious across a broad spectrum of PH patients, a disease underpinned by several, disparate pathogenic mechanisms.

As would be predicted by previous work, dietary nitrate reduced systemic blood pressure in parallel to its effects on pulmonary hemodynamics. However, the relative change in pulmonary versus systemic pressures implies that it is possible to obtain a certain degree of selectively for the pulmonary vasculature utilizing this approach; at very least, it should be possible to bring about a significant and efficacious reduction in pulmonary artery pressure whilst maintaining acceptable systemic pressure. Indeed, dietary nitrate is likely to be superior to administration of NO-donors in the treatment of PH which, despite the considerable success with inhaled NO in persistent PH of the newborn[9], have shown limited efficacy[47, 48]; this is predominantly due to lack of pulmonary selectivity (only achieved with inhalation thereby posing logistical difficulties with delivery), potential for rebound PH, and ubiquitous cGMP-independent cytotoxic effects. Our data, therefore, give credence to the idea that dietary nitrite, and perhaps more favorably dietary nitrate, may offer therapeutic benefit in the treatment of PH. Since dietary nitrate can be easily and inexpensively (self-)

administered by ingestion of green leafy vegetables, this strategy could be evaluated rapidly and offer a low-cost advance in treating PH (with the caveat that a combination treatment with PDE5i and dietary nitrate does not cause unacceptable systemic hypotension, given treatment with organic nitrates and PDE5i is contraindicated). Moreover, in emerging economies in which PH is associated with hypoxia (altitude) or high incidence of lung disease, where current therapy may not be readily available or affordable, dietary nitrate may offer a genuine alternative.

Definitive evidence for a mammalian nitrate reductase remains elusive and genomic comparison suggests that mammalian homologues of classical bacterial nitrate reductases do not exist, although there is some evidence that XOR may act as a mammalian nitrate reductase[18, 49]. It is generally accepted that facultative anaerobes on the dorsal surface of the tongue are responsible for the endogenous conversion of nitrate to nitrite, which is subsequently reduced to bioactive NO by enzymes including XOR[18-20], globins[22-24], and aldehyde oxidase[25]. A further possibility lies with the utilization of nitrite as a substrate by eNOS[26, 27], to maintain NO output under conditions of hypoxia when conventional NO synthesis from L-arginine is impaired. Intriguingly, evidence suggests that eNOS expression is increased in PH (despite the compromised NO bioavailability & bioactivity)[50]. Thus, in the hypoxic environment associated with PH, eNOS might be up-regulated as a compensatory mechanism in an attempt to offset the hemodynamic dysfunction; perhaps as a means to utilize nitrite, rather than L-arginine, to synthesize NO.

In order to determine if eNOS may be responsible for the beneficial effects of dietary nitrate in PH, we employed eNOS KO animals in the same model of hypoxia-induced PH. In these animals, when compared to WT littermates, the positive effects of dietary nitrate (and nitrite) on cardiopulmonary hemodynamics and morphology were significantly reduced. Such a clear loss of efficacy confirms that the nitrite reductase activity of eNOS underpins the pharmacodynamic effects of inorganic nitrite and nitrate (at least in this model). These data therefore highlight a key functional switch that eNOS undergoes to enable utilization of alternate substrates (i.e. L-arginine versus nitrite) under changing local environments (e.g. normoxia versus hypoxia) to maintain the output of cytoprotective NO.

Intriguingly, some residual efficacy of dietary nitrate remains in eNOS KO animals, suggesting that alternative mechanism(s) for reduction of nitrite persist. One potential candidate is XOR, which has been demonstrated to act as a nitrite reductase in animal models of PH[34]. In order to ascertain if this enduring activity may be the result of XOR-catalyzed nitrite reduction, additional studies were conducted using hypoxia-induced PH in the presence of the XOR inhibitor allopurinol. Here, the beneficial effects of dietary nitrate were also reduced; indeed, the loss of efficacy was very similar to that observed in eNOS KO mice. These data intimate that, in addition to eNOS, XOR plays a central nitrite reductase function in the hypoxic environment associated with PH. This thesis is also supported by *in vitro* biochemical assessment of the lung nitrite reductase activity from animals with hypoxia-induced PH. In these tissues, addition of nitrite resulted in a concentration-dependent increase in the generation of free NO that was blocked by either L-NMA or allopurinol.

Whilst our data largely advocate the administration of either inorganic nitrite or nitrate for the treatment of PH, it appears that nitrate would be the preferred species. Only nitrate was able to reduce the severity of all indices of disease progression that were assessed, including RVSP, RVH and pulmonary vascular remodeling. This is probably due to a more prolonged and maintained pharmacokinetic profile of nitrite production compared with the direct ingestion of nitrite, which appears to be rapidly excreted and has a short plasma half-life)[34-36]. Nonetheless, previous work demonstrating the nebulization of inorganic nitrite

has acute effects on pulmonary hemodynamics and development of PH[32, 34] gives credence to the possibility of using this mode of delivery in treating PH. Undoubtedly, this approach is easier than administration of NO via inhalation, but remains suboptimal in terms of efficiency of delivery and compliance. The use of dietary changes to increase ingestion of inorganic nitrate (and/or nitrite), or the use of a pill, represents a modality of therapy that could be rapidly and cost-effectively implemented to treat PH.

Acknowledgments

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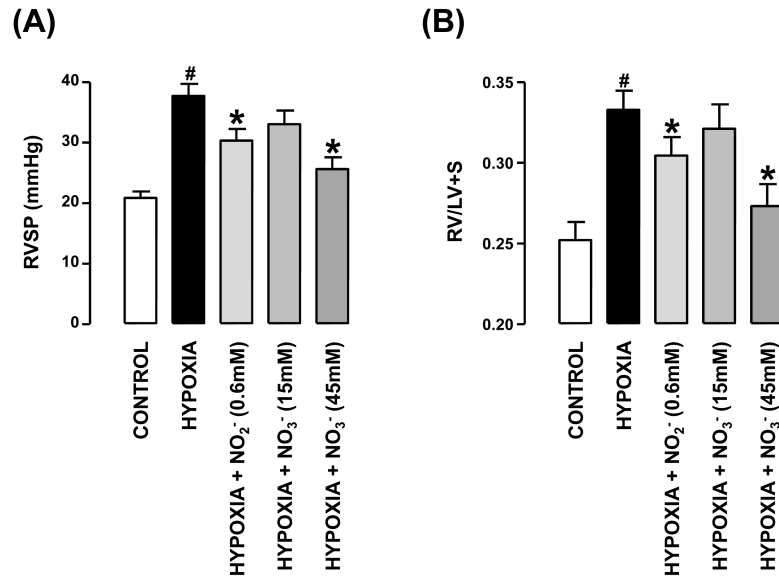
(A)

(B)

Figure 1.
(A) Right ventricular systolic pressure (RVSP) and **(B)** right ventricle:left ventricle plus septum ratio (RV/LV+S) in normoxic (control) WT mice and WT animals exposed to 3 weeks hypoxia (10% O_2) in the absence and presence of inorganic nitrite (NO_2^-; 0.6mM) or inorganic nitrate (NO_3^-; 15mM or 45mM). [#]$P<0.05$ v normoxia; *$P<0.05$ versus hypoxia. n=17-25 for each group.
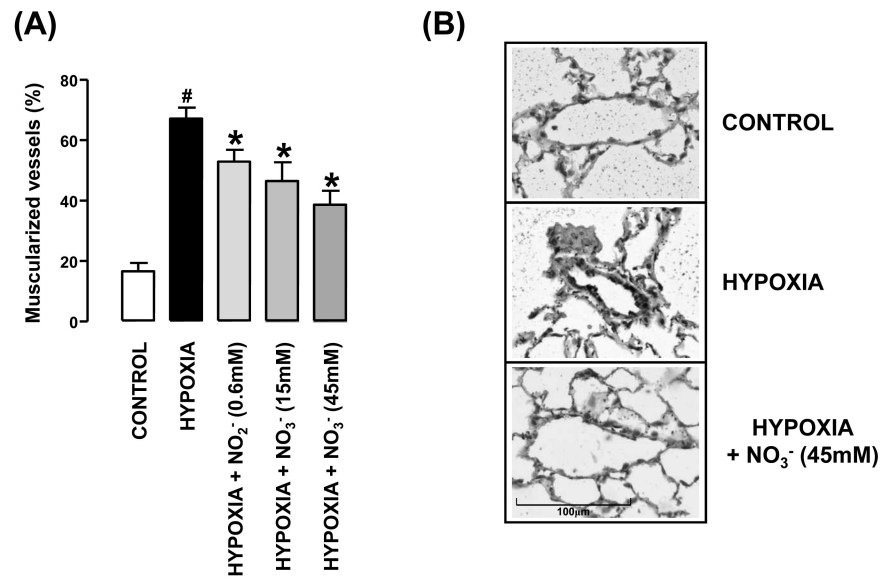
Figure 2.
(A) % Muscularized vessels in normoxic (control) WT mice and WT animals exposed to 3 weeks hypoxia (10% O_2) in the absence and presence of inorganic nitrite (NO_2^-; 0.6mM) or inorganic nitrate (NO_3^-; 15mM or 45mM). #$P<0.05$ v normoxia; *$P<0.05$ versus hypoxia. n=17-25 for each group. **(B)** Representative light-microscopic images (80× magnification) of pulmonary arteries from normoxic, hypoxic and nitrate (45mM)-treated animals; the hypoxic vessels exhibit a marked muscularization that is reduced in the presence of nitrate (45mM).
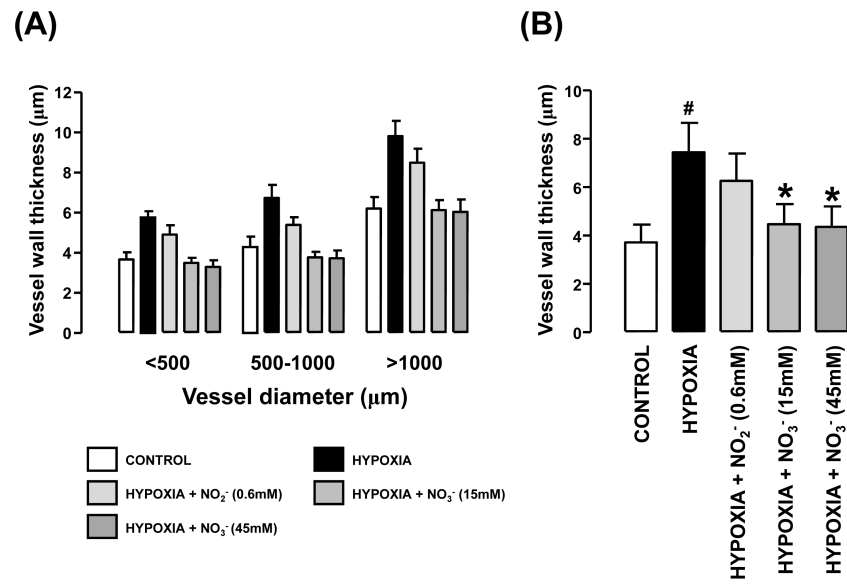
(A)

(B)

Figure 3.
Vessel wall thickness in **(A)** subpopulations and **B)** all arteries in the pulmonary circulation of normoxic (control) WT mice and WT animals exposed to 3 weeks hypoxia (10% O_2) in the absence and presence of inorganic nitrite (NO_2^-; 0.6mM) or inorganic nitrate (NO_3^-; 15mM or 45mM). [#]$P<0.05$ v normoxia; *$P<0.05$ versus hypoxia. n=120 for each group.
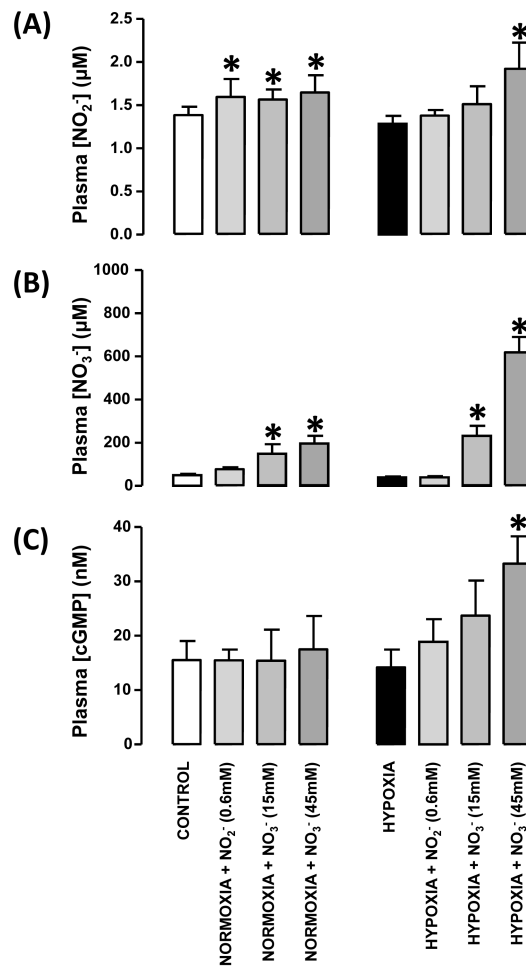
Figure 4.
Plasma (**A**) nitrite (NO_2^-), (**B**) nitrate (NO_3^-) and (**C**) cGMP concentrations in normoxic (control) WT mice and WT animals exposed to 3 weeks hypoxia (10% O_2) in the absence and presence of inorganic nitrite (NO_2^-; 0.6mM) or inorganic nitrate (NO_3^-; 15mM or 45mM). [#]P<0.05 v normoxia; *P<0.05 versus hypoxia. n=12-16 for each group.
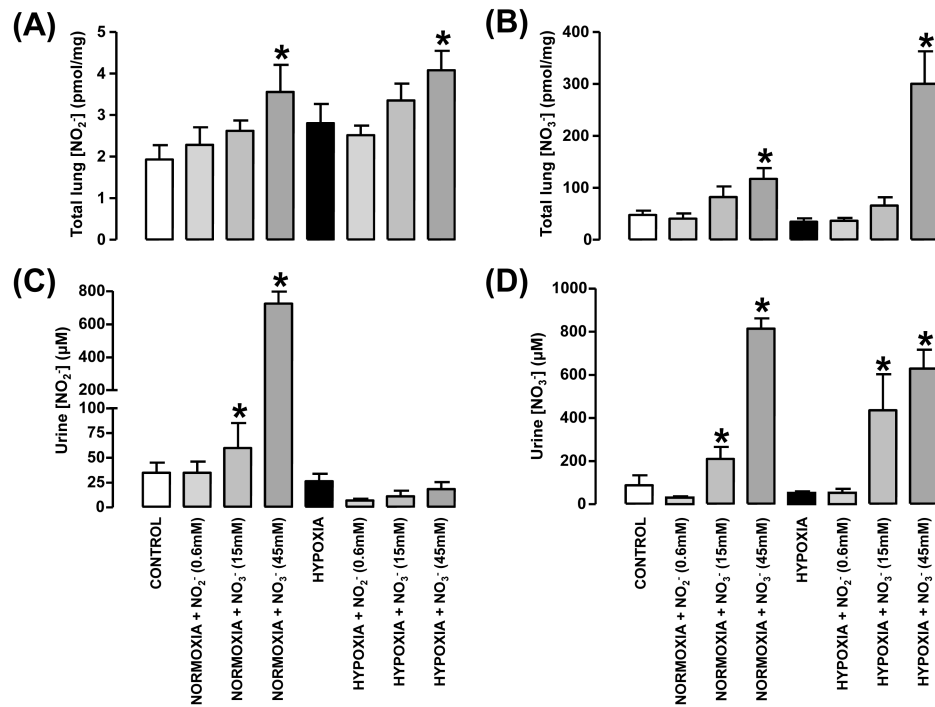
Figure 5.
Total lung and urinary nitrite (NO_2^-) **(A & C)** and nitrate (NO_3^-) **(B & D)** concentrations in normoxic (control) WT mice and WT animals exposed to 3 weeks hypoxia (10% O_2) in the absence and presence of inorganic nitrite (NO_2^-; 0.6mM) or inorganic nitrate (NO_3^-; 15mM or 45mM). *$P<0.05$ versus hypoxia. n=12-16 for each group.
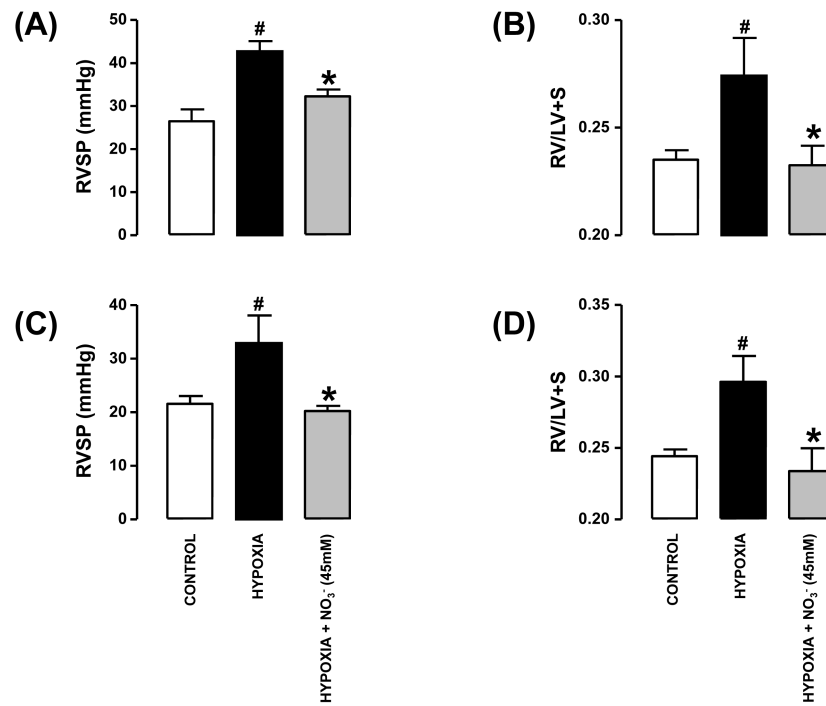
Figure 6.
Right ventricular systolic pressure (RVSP) and right ventricle:left ventricle plus septum ratio (RV/LV+S) in normoxic (control) WT mice and WT animals exposed to 5 weeks hypoxia (10% O_2) **(A & B)** or bleomycin (1mg/kg) **(C & D)** in the absence and presence of inorganic nitrate (NO_3^-; 45mM). Inorganic nitrate was administered at weeks 3-5 in the hypoxic studies and weeks 0-3 in the bleomycin model. [#]P<0.05 v normoxia; *P<0.05 versus hypoxia. n=6-10 for each group.
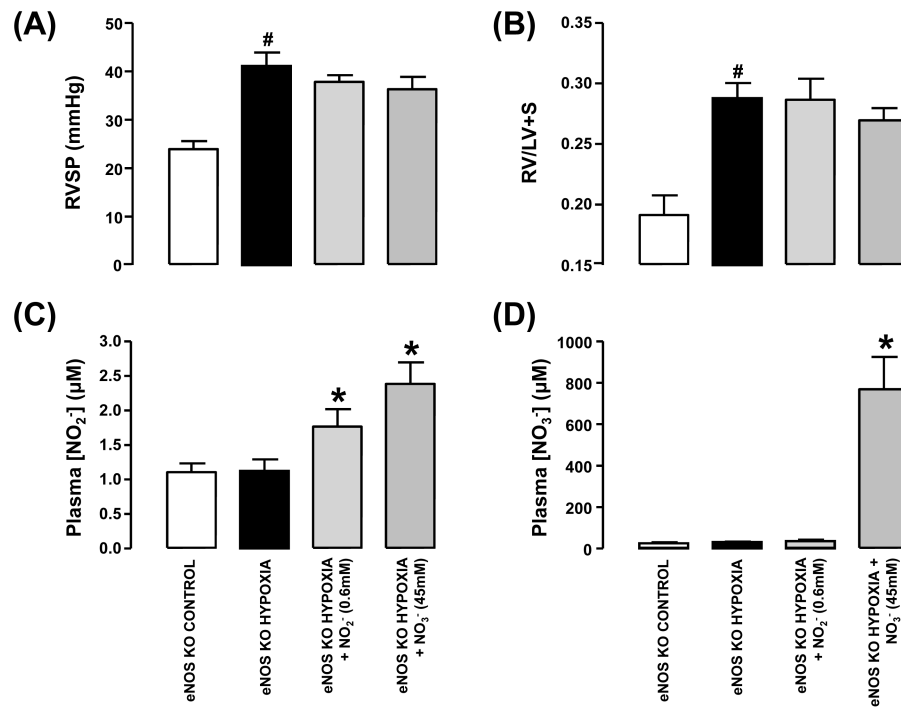
Figure 7.
Right ventricular systolic pressure (RVSP) **(A)**, right ventricle:left ventricle plus septum
ratio (RV/LV+S) **(B)**, and plasma nitrite (NO_2^-) **(C)** and nitrate (NO_3^-) **(D)** concentrations
in eNOS KO normoxic mice and eNOS KO animals exposed to 3 weeks hypoxia (10% O_2)
in the absence and presence of inorganic nitrite (NO_2^-; 0.6mM) or inorganic nitrate (NO_3^-;
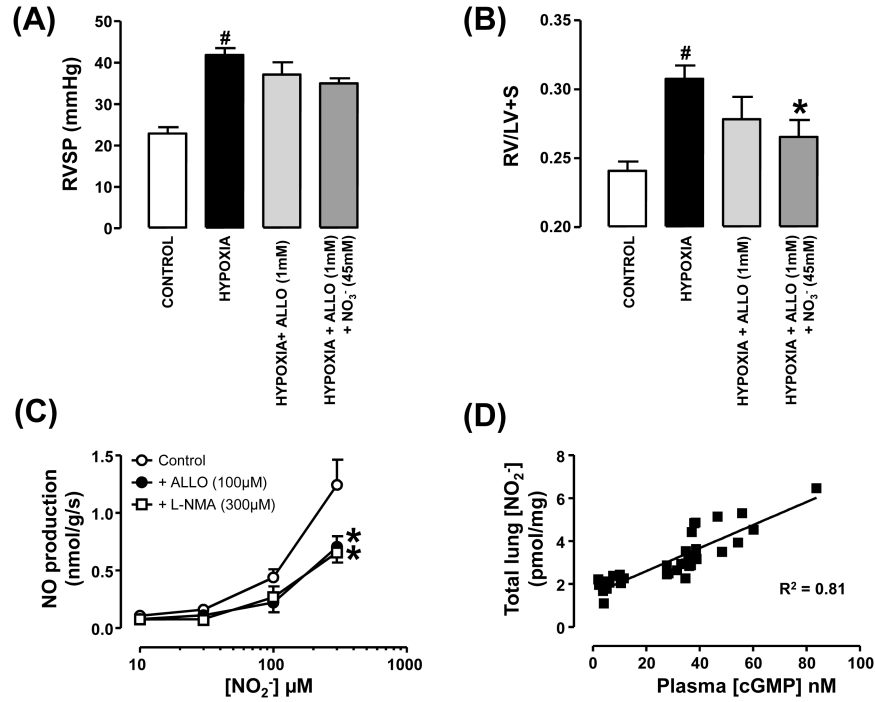45mM). [#]P<0.05 v normoxia; *P<0.05 versus hypoxia. n=6-12 for each group.

Figure 8.
Right ventricular systolic pressure (RVSP) **(A)**, right ventricle:left ventricle plus septum ratio (RV/LV+S) **(B)** in normoxic mice and animals exposed to 3 weeks hypoxia (10% O_2) in the absence and presence of inorganic nitrate (NO_3^-; 45mM) and allopurinol (1mM). [#]P<0.05 v normoxia; *P<0.05 versus hypoxia. n=6-12 for each group. Lung nitrite reductase activity in response to sodium nitrite (10-300μM) in the absence and presence of L-NMA (300μM) or allopurinol (100μM) **(C)**. *P<0.05 versus control across entire curve. n=3-5 for each group. Correlation between lung nitrite (NO_2^-) and plasma cGMP concentrations *[D]*. n=34.

Table 1

Amount of nitrite (NO_2^-) and nitrate (NO_3^-) consumed by wild type (WT) and eNOS knockout (KO) animals during exposure to hypoxia (10% O_2; 3 weeks). Values are expressed as mean±s.e.m; n 8.

	H$_2$O (ml) consumed/day	Amount NO$_2^-$ consumed (mg/kg/day)	Amount of NO$_3^-$ consumed (mg/kg/day)
WT: Normoxia	4.58±0.13	<0.001	<0.001
WT: Hypoxia	4.15±0.12	<0.001	<0.001
WT: Hypoxia + NO$_2^-$ (0.6mM)	4.11±0.09	4.35±0.05	<0.001
WT: Hypoxia + NO$_3^-$ (15mM)	4.47±0.15	<0.001	155.44±5.24
WT: Hypoxia + NO$_3^-$ (45mM)	4.14±0.41	<0.001	431.91±43.20
KO: Normoxia	4.30±0.31	<0.001	<0.001
KO: Hypoxia	4.27±0.11	<0.001	<0.001
KO: Hypoxia + NO$_2^-$ (0.6mM)	4.43±0.11	4.68±0.03	<0.001
KO: Hypoxia + NO$_3^-$ (45mM)	4.20±0.18	<0.001	438.48±18.40

Table 2

Effect of normoxia, hypoxia (10% O_2; 3 weeks), and dietary nitrite (NO_2^-) and nitrate (NO_3^-) supplementation on mean arterial blood pressure (MABP) in wildtype (WT) and eNOS knockout (eNOS KO) mice. Values are expressed as mean±s.e.m; n 8.

	WT MABP (mmHg)	eNOS KO MABP (mmHg)
Normoxia	104.4±5.7	127.3±7.3
Hypoxia	108.3±8.2	120.0±2.9
Hypoxia + **NO_2^- (0.6mM)**	104.1±9.4	118.8±2.7
Hypoxia + **NO_3^- (15mM)**	100.1±4.5	n.d.
Hypoxia + **NO_3^- (45mM)**	94.5±2.6 [*]	118.7±2.6

[*] $P < 0.05$ versus hypoxia only;

n.d., not determined