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Inhaled nebulized nitrite and nitrate therapy in a canine model of hypoxia-induced pulmonary hypertension

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A B S T R A C T

Dysfunction in the nitric oxide (NO) signaling pathway can lead to the development of pulmonary hypertension (PH) in mammals. Discovery of an alternative pathway to NO generation involving reduction from nitrate to nitrite and to NO has motivated the evaluation of nitrite as an alternative to inhaled NO for PH. In contrast, inhaled nitrate has not been evaluated to date, and potential benefits include a prolonged half-life and decreased risk of methemoglobinemia. In a canine model of acute hypoxia-induced PH we evaluated the effects of inhaled nitrate to reduce pulmonary arterial pressure (PAP). In a randomized controlled trial, inhaled nitrate was compared to inhaled nitrite and inhaled saline. Exhaled NO, PAP and systemic blood pressures were continuously monitored. Inhaled nitrite significantly decreased PAP and increased exhaled NO. In contrast, inhaled nitrate and inhaled saline did not decrease PAP or increase exhaled NO. Unexpectedly, we found that inhaled nitrite resulted in prolonged (> 5 h) exhaled NO release, increase in nitrate venous/arterial levels and a late surge in venous nitrite levels. These findings do not support a therapeutic role for inhaled nitrate in PH but may have therapeutic implications for inhaled nitrite in various disease states.

1. Introduction

Pulmonary arterial hypertension is a proliferative vasculopathy affecting the small pulmonary arteries resulting in progressive pulmonary vascular remodeling, increased pulmonary vascular resistance, and eventually, right-sided heart failure, reduced cardiac output and death [1]. Imbalances between vasodilators and vasoconstrictors, growth inhibitors and mitogenic factors, and antithrombotic and prothrombotic elements appear to underlie these pathophysiological changes [1,2].

Nitric oxide (NO) has a key role in maintaining cardiovascular homeostasis [3–7]. Reduced NO bioavailability is a hallmark in numerous cardiovascular disorders, including pulmonary arterial hypertension. NO produced in the pulmonary vascular endothelium diffuses to smooth muscle mediating vascular vasodilatory, antiproliferative and antithrombotic effects [1,2]. Numerous therapies targeting the pathway have been studied, and some have been proved effective in the treatment of pulmonary arterial hypertension. Inhaled NO is currently FDA-approved for the treatment of persistent pulmonary hypertension in newborns [8] and it has also been evaluated in older children and adults with pulmonary hypertension [9–13]. However, the initial enthusiasm for this therapeutic approach has weakened due to practical and economic factors associated with its administration. Therefore, several attempts have been made to use alternative drugs that result in NO release.

Two major pathways contribute to NO generation in mammals [14]; the well-established L-arginine-NO synthase-NO (NOS) pathway [15], and the recently discovered nitrate-nitrite-NO reduction pathway [14,16]. This alternative pathway to generate NO involves reductions of nitrate to nitrite and then nitrite to NO, and is suggested to be involved in many important biological processes, including regulation of blood flow and responses to hypoxia [14]. Nitrite reduction to NO *in vivo* has been shown to occur in several organs as well as in the blood. Numerous mammalian enzymatic pathways have been involved in nitrite reduction to NO, including haemproteins, such as deoxygenated hemoglobin [17], neuroglobin, and myoglobin [17–22]; molybdopterin proteins, such as xanthine oxidoreductase (XOR) and aldehyde oxidase (AO) [23–27]; carbonic anhydrase [28] and even NOS [29]. In contrast, the reduction of nitrate to nitrite independent of commensal bacteria in the enterosalivary pathway is less well understood. Although mammals do not have specific dedicated nitrate reductases, the reduction of nitrate to nitrite through non-specific enzymatic pathways has been recently shown to occur *in vitro* and *in vivo* in several organs [30–35]. Most studies have suggested that the mammalian reduction of nitrate to nitrite is most likely mediated by the molybdopterin proteins XOR and AO

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[30,36].

The discovery of the nitrate-nitrite-NO pathway has led to nitrite and nitrate being investigated as therapeutic agents in lieu of NO, using a variety of administration routes including oral, inhaled and intravenous [37–39]. In particular, inhaled nebulized nitrite has been evaluated as an alternative to inhaled NO for pulmonary hypertension in animal models [40–43] and humans [44–46]. Safety, tolerability, and the pharmacokinetics have been extensively studied [44], and mechanisms of action proposed [47,48]. The main safety concern is that prolonged use of nitrite leads to increased methemoglobin, due to its reaction with oxyhemoglobin. In addition, inhaled nitrite, similarly to inhaled NO, has short-lived effects that disappear soon after discontinuing the therapy.

In contrast to nitrite, a direct therapeutic approach, nitrate has been mostly considered as a "pro-drug" therapeutic intervention. Studies using nitrate have focused on increasing its dietary intake, thus targeting its entero-salivary conversion to nitrite, as this is known to be the main pathway for nitrate reduction to nitrite and to NO in mammals [5,49–53]. Dietary nitrate has been successfully used to decrease pulmonary hypertension in mice [39], which could lead to a supportive long-term therapy. Recently, oral nitrate-rich beetroot juice supplementation was tested in an exploratory randomized, placebo-controlled crossover trial of 15 patients with pulmonary hypertension [54] and additional clinical trials using dietary nitrate interventions for are currently ongoing [55]. However, in the acute setting requiring rapid decrease in pulmonary pressures, dietary nitrate would not likely replace direct nitrate delivery into the respiratory system. To date, specific organ-targeted nitrate therapy has not been evaluated. It is now known that nitrate can be reduced to nitrite in mammalian tissues independently of the entero-salivary circulation and the presence of commensal bacteria [30,31,35,36]. The degree to which this reduction occurs *in vivo* is not well established, as well as whether nitrate interventions targeted to specific organs may represent a feasible and useful approach. The longer half-life of nitrate (~5–6 h) [56] and slower reduction rate to nitrite may actually represent a potential advantage of this approach by allowing a more progressive and prolonged activation of NO signaling pathway.

Here, we hypothesized that inhaled nitrate can be used as a precursor of NO to lower pulmonary pressures in a canine model of hypoxia-induced acute pulmonary hypertension. If successful, nitrate has a better risk/benefit therapeutic profile compared to nitrite due to its non-reactivity with hemoglobin and decreased methemoglobin formation, as well as its known circulation half-life of several hours, predicting therapeutic effects could be lasting well past inhalation termination. To our knowledge, the efficiency of nitrate in lung tissue has not been systematically studied, even if enzymes with known nitrate reductase activity- XOR and AO -are expressed in lung tissue [57–59].

2. Methods

All large animal studies described were approved by the National Institutes of Health Clinical Center Institutional Animal Care and Use Committee (Protocol CCM 16–04). All small animal procedures were carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of NIH under Animal Care and Use Committee approved protocol (Protocol NIDDK K-049-17).

Three experiments were conducted in large animals using nineteen 10-to-12-kg, one-to-two-year-old purpose-bread beagles. First, using a single animal, we developed a hypoxia and vasoconstriction-induced pulmonary hypertension model. Next, a dose-finding study varying inhaled doses of aerosolized nitrite, nitrate and saline (control) was done with nine other animals. Finally, a small randomized controlled trial was conducted to compare inhaled aerosolized nitrite (positive control), nitrate and saline (negative control) in a model of hypoxia-induced pulmonary hypertension in nine additional animals. An additional experiment was conducted in adult Wistar rats (n = 2, weight 250 ± 50 g, Charles River Laboratories, Wilmington, MA) to obtain tissue for Western blots.

2.1. Large animal preparation

All large animal experiments described were performed under deep sedation or general anesthesia. Specifically, anesthesia was induced via mask inhalation using isoflurane (1%–5%) and the animals were then intubated (6 mm, Rusch, Duluth, GA) and mechanically ventilated (Servo-I, Maquet, Wayne, NJ); fractional inspired oxygen 50% during procedures, positive end expiratory pressure of 5 cm H_2O; ventilation rate at 15 breaths/min; and tidal volume of 20 ml/kg as previously described [60].

Animals were then instrumented using aseptic techniques with peripheral catheters; femoral arterial (20 gauge), external jugular venous (8-French), radial venous (18-gauge) catheters (Maxxim Medical, Athens, TX), and urinary drainage catheters (Cook Medical, Foley 8 Fr, 55 cm, Bloomington, IN) were placed. A tracheostomy was surgically performed, and a breathing tube placed (Covidien, Shiley 6LPC, Mansfield, MA) to maintain a secure airway during prolonged mechanical ventilation with sedation as previously described [60]. A pulmonary artery thermodilution catheter (7- French, Abbott Critical Care, Chicago, IL) was later introduced through the external jugular vein introducer to continuously measure pulmonary artery pressures (PAP).

After catheter placement, the anesthetic gas was discontinued and continuous infusions of midazolam (2.5–5 mg/kg/min infusion) and fentanyl (0.16 mg/kg/min infusion) were initiated and maintained for the duration of the study. The mechanical ventilation parameters were unchanged except the fractional inspired oxygen was lowered to 21%. After a steady state period breathing room air, baseline measures are performed, and the study initiated.

Throughout the study, mean (mPAP) and pulmonary (PAP) arterial and central venous pressure (CVP) were measured continuously, and pulmonary capillary wedge pressure (PAOP) and cardiac output (CO) were measured intermittently at pre-specified time points. Exhaled NO concentration was measured continuously at the first 5 cm of the exhalation tube with a chemiluminescence NO analyzer (NOA 280, Sievers Instruments, Inc.).

Animals were continuously monitored during the study for signs of distress and the infusions of narcotics and sedatives were adjusted appropriately according to established protocols. After the experiments were completed, while still sedated, all animals were euthanized (Euthanasia – D 75 mg/kg of Sodium Pentobarbital (390 mg/ml) IV).

2.2. Small animal preparation

All small animal experiments where conducted initially in an enclosed anesthesia box using 5% isoflurane mixture with air. Anesthetized animals were then placed on a pad in supine position and anesthesia continued through a nose cone. The thoracic cavity was opened and ~9–10 ml of blood collected by cardiac puncture, representing about two-thirds of total blood volume for an animal of this size. Animals were then perfused using heparin-containing saline to flush the remaining blood out of tissues. Perfusion continued until no blood was detected in outgoing saline and liver and kidneys were discolored. Samples from liver and skeletal muscle from hind leg were collected after perfusion and flash frozen on dry ice for the Western blot. All samples were stored at −80 °C until analysis. Animals were housed in a 12-h light/dark cycle environment with access to food and drinking water *ad libitum*.

2.3. Large animal hypoxia and vasoconstriction-induced pulmonary hypertension model development

We studied an initial single animal to test the hypothesis that

hypoxia with or without U46619, a stable synthetic analog of the endoperoxide prostaglandin PGH 2 which acts as a thromboxane A 2 (TP) receptor agonist (0.3–0.6 mg/kg/min IV; Tocris, Minneapolis MN), can induce an increase in PAP of at least 150% from baseline. After baseline measurements breathing room air were obtained, pulmonary hypertension was successfully achieved and maintained during a 210-min period where the FiO2 of inspired gas was decreased to 15% (85% balance was nitrogen). The rise in pulmonary pressure occurred within the first 5–10 min of the initial 20-min period with hypoxia alone. The U46619 was added for 20 min and further increased pulmonary pressures. PAP was measured serially every 5 min and was maintained between 150 and 200% of the baseline pressure with hypoxia and U46619. At the end of the period of hypoxia, FiO2 was increased to 21% and U46619 was discontinued, resulting in a quick reversal of pulmonary hypertension.

2.4. Large animal dose-response with inhaled aerosolized nitrite, nitrate and saline

We first studied six animals to test the hypothesis that inhaled nitrite can reverse induced pulmonary hypertension in canines. We planned that if inhaled nitrite does reverse experimental pulmonary hypertension in this model, we would select the lowest effective dose of inhaled nitrite to study. After baseline measurements breathing room air were obtained, FiO2 of inspired gas was decreased to 15% for a period of approximately 40 min during which pulmonary hypertension was induced and maintained at a PAP between 150 and 200% of baseline. One of the three animals randomized to receive nitrite did not reach the target range during the initial 20-min period and a U46619 infusion was added to reach the target PAP. After the period of hypoxia-induced pulmonary hypertension, increasing doses of aerosolized nitrite was inhaled (30 mg/min, 60 mg/min, 120 mg/min, 240 mg/min and/or 300 mg/min, incremented every 20 min). In three animals randomized as controls, a similar volume of aerosolized saline was administered in lieu of nitrite.

Subsequently, we studied three additional animals in a dose-finding study to determine an effective dose of inhaled nitrate. With a similar design as described above, all animals were exposed to a period of hypoxia after the initial baseline measurements were obtained. After 40 min of hypoxia in which only one of the three animals also received U46619, the three animals inhaled increasing doses of aerosolized nitrite (nitrate) (30 mg/min, 60 mg/min, 120 mg/min, 240 mg/min and/or 300 mg/min) incremented every 20 min.

2.5. Large animal pilot randomized controlled trial

We studied nine animals in a randomized controlled trial design comparing inhaled aerosolized nitrite, nitrate and saline in our large animal model of hypoxia-induced pulmonary hypertension. The dose (mg/min) selected was based on the results of the dose-response of the prior inhaled nitrite study. Nitrite alone, but not saline or nitrate, had an effect on pulmonary pressures in response to increasing inhaled dose. U46619, used to raise PAP, was discontinued for use in this study due to transient gastrointestinal bleeding in animals during the dose-finding study.

The randomized trial was divided into five periods: 1) baseline (20 min breathing room air); 2) hypoxia-induced pulmonary hypertension (40 min at FiO2 = 15%); 3) inhaled therapy (30 min) while continuing the hypoxia-induced pulmonary hypertension (FiO2 = 15%); 4) 2-h after end of inhaled therapy (acute effects) while continuing the hypoxia-induced pulmonary hypertension (FiO2 = 15%) and; 5) 4-h after end of inhaled therapy (delayed effects) while continuing the hypoxia-induced pulmonary hypertension (FiO2 = 15%). Described briefly, after obtaining baseline measurements breathing room air (and repeated at the end of each period), animals were randomized to either inhaled nitrite, nitrate or saline (3 animals per group). FiO2 of inspired gas was decreased to 15% for a period of 40 min to induce pulmonary hypertension. After this period, inhaled therapies were initiated and maintained for 30 min. For animals receiving nitrite or nitrate, we administered the inhaled therapy at a single dose (30 mg/min) that had proven effective in our nitrite dose-response study to elicit a sustained physiologic response without side effects. Animals randomized to receive saline were administered equivalent volumes of inhaled saline (15 ml) during this period. After 30 min, inhaled therapies were terminated, and the animals were followed for 24 h from the beginning of the study.

During the first three periods of the study, mean PAP and exhaled NO were measured and recorded every 5 min. After inhaled therapies ended, these measurements were recorded every 5 min for the next hour and every 30 min thereafter.

2.6. Delivery of nebulized nitrite, nitrate or saline in large animals

Nitrate and nitrite solutions for the study were prepared fresh for each study day. Sodium nitrite or sodium nitrate (Sigma-Aldrich St. Louis, MO) were dissolved in buffered saline (K-D Medical Columbia, MD) at increasing concentrations. Nitrite and nitrate solutions were administered using a jet nebulizer (Aerogen, Solo, Galway, IR) which delivered a constant rate of 30 mg/min for 30 min except during the dose-response study as described above. Nebulized nitrite and nitrate were delivered via the inspiratory loop of the ventilator.

2.7. Measurement of plasma nitrite and nitrate, and exhaled NO in large animals

Arterial and venous nitrite and nitrate samples were obtained from femoral arterial and central venous catheters respectively. Plasma was separated by centrifugation and immediately frozen on dry ice and stored at −80 °C until assayed using the chemiluminescence methodologies using the NO analyzer (model 280i NO analyzer, Sievers, Boulder, CO) as previously described [61]. These samples were obtained at baseline and at the end of each of the five experimental periods described above.

2.8. Western Blots in large and small animals

Animal tissues for Western blotting were obtained from stored samples of normal controls from previous approved studies (canine protocol number CCM16-04, rat protocol number K049-MMB-17). Canine and rat liver and lung homogenates were prepared by GentleMacs dissociator (Miltenyi Biotec, USA) with 10 mM Tris-Cl (pH 7.4) containing 250 mM sucrose. Protein concentration of the homogenates was determined by BCA assay. Denatured protein samples (50 μg) were run on SDS-PAGE, then transferred to nitrocellulose membrane. The membrane was incubated with XOR antibody (Abcam, ab133268) overnight at 4 °C, then with anti-rabbit IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch, 111-035-144). XOR band was detected by ECL (Thermo Scientific, 34095).

2.9. Statistical methods

We used linear mixed models to build contrasts for various comparisons and account for repeated measurements of each animal and the actual pairing of animals within each cycle. Standard residual diagnostics were used to check model assumptions, and data were log-transformed when necessary. SAS version 9.4 (Cary, NC) was used for all analyses. All p-values are two-tailed and considered significant if $p \leq 0.05$.

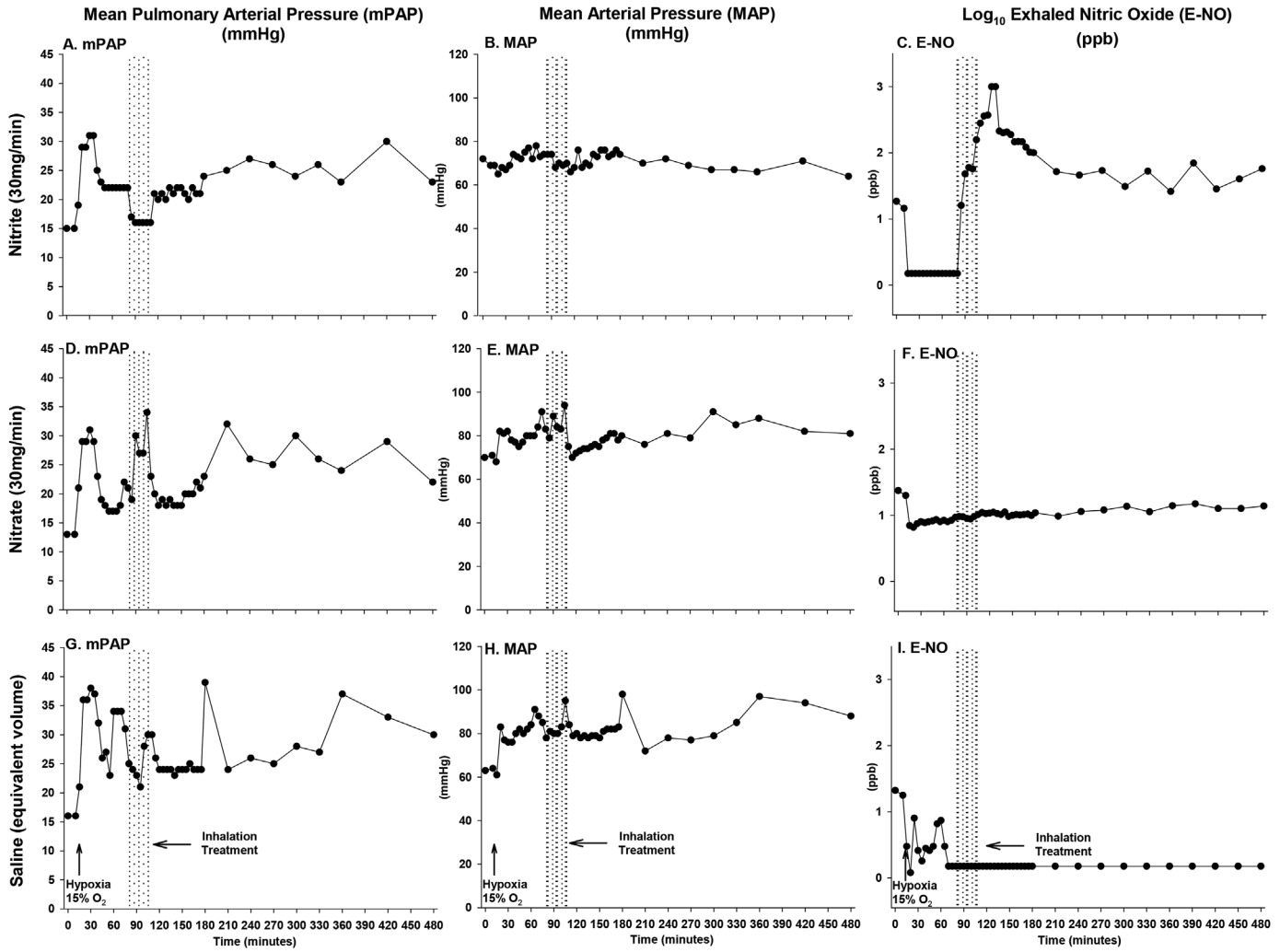

Fig. 1. Illustrative examples of effects of inhaled study drugs on vascular tone and exhaled nitric oxide production. The time interval of the inhalation treatment of nitrite (Panel A, B and C), nitrate (Panel D, E and F) and saline (Panel G, H and I) is depicted in each panel by vertical dashed and dotted lines. The time on the x-axis from 0 to 480 min when hypoxia was initiated is shown by a vertical arrow and inhalation treatment was given by a horizontal arrow. Time is plotted on the x-axis verses on the y-axis the mPAP (Panel A, D and G), MAP (Panel B, E and H) and log[10] exhaled NO levels. For unlogged exhaled NO values please see supplementary e-Fig. 1.

3. Results

3.1. Randomized controlled trial: individual serial responses to therapies

Fig. 1 shows the serial mPAP (far left panels), MAP (middle panels) and exhaled NO levels responses of three individual animals representative of the three treatment groups (inhaled nitrite, inhaled nitrate and inhaled saline) from the randomized trial (see Supplementary Fig. 1 for non-logarithmic exhaled NO data) throughout different phases of the experiment: 1) breathing room air, 2) breathing 15% oxygen/85% nitrogen gas mixture, 3) breathing 15% oxygen/ 85% nitrogen and receiving treatment with either inhaled nitrite (30 mg/min, top panels) or nitrate (30 mg/min, middle panels) or saline (15 ml equivalent volume, bottom panels) for 30 min. All three animals had elevations in mPAP after changing from breathing room air to 15% oxygen. Initiation of inhaled nitrite therapy resulted in a rapid fall in mPAP that reversed upon termination of the inhaled treatment. The two animals receiving the other two treatments (inhaled nitrate and saline) did not have similar decreases in mPAP. A large spike in measured exhaled NO levels occurred following inhaled nitrite treatment but there were no elevations in measured exhaled NO in the other two treatments. Differences in MAP were not pronounced enough to make inferences from the individual data for the three animals. These data suggest that at this dose inhaled nitrite but not nitrate can be converted to NO, resulting in lowering the elevated mPAPs secondary to hypoxia.

3.2. Randomized controlled trial: summary of hemodynamic and exhaled NO data

Fig. 2 shows the summary data for the nine animals receiving the same three treatments shown in Fig. 1, where each treatment was given to three of the nine animals (see Supplementary Fig. 2 for non-logarithmic exhaled NO data). Animals from all three groups had significant elevations in mPAP in response to reducing oxygen concentration from 21% (room air) to 15% oxygen ($p = 0.02$ to $p = 0.002$). Table 1 shows the serial mean PaO_2 changes in those animals, as well as other hemodynamic parameters. Notably, only the animals receiving inhaled nitrite (and not those receiving the other two inhalation therapies, all, $p > 0.05$) had a statistically significant fall in mPAP and MAP ($p = 0.03$ and $p = 0.02$, respectively) during treatment. Nitrite inhalation resulted in significant elevations in mean exhaled NO levels during and for 4 h following treatment ($p = 0.0005$ to $p < 0.0001$). Treatment with inhaled nitrate or saline resulted in no significant elevations in mean exhaled NO levels during or after treatment. These data

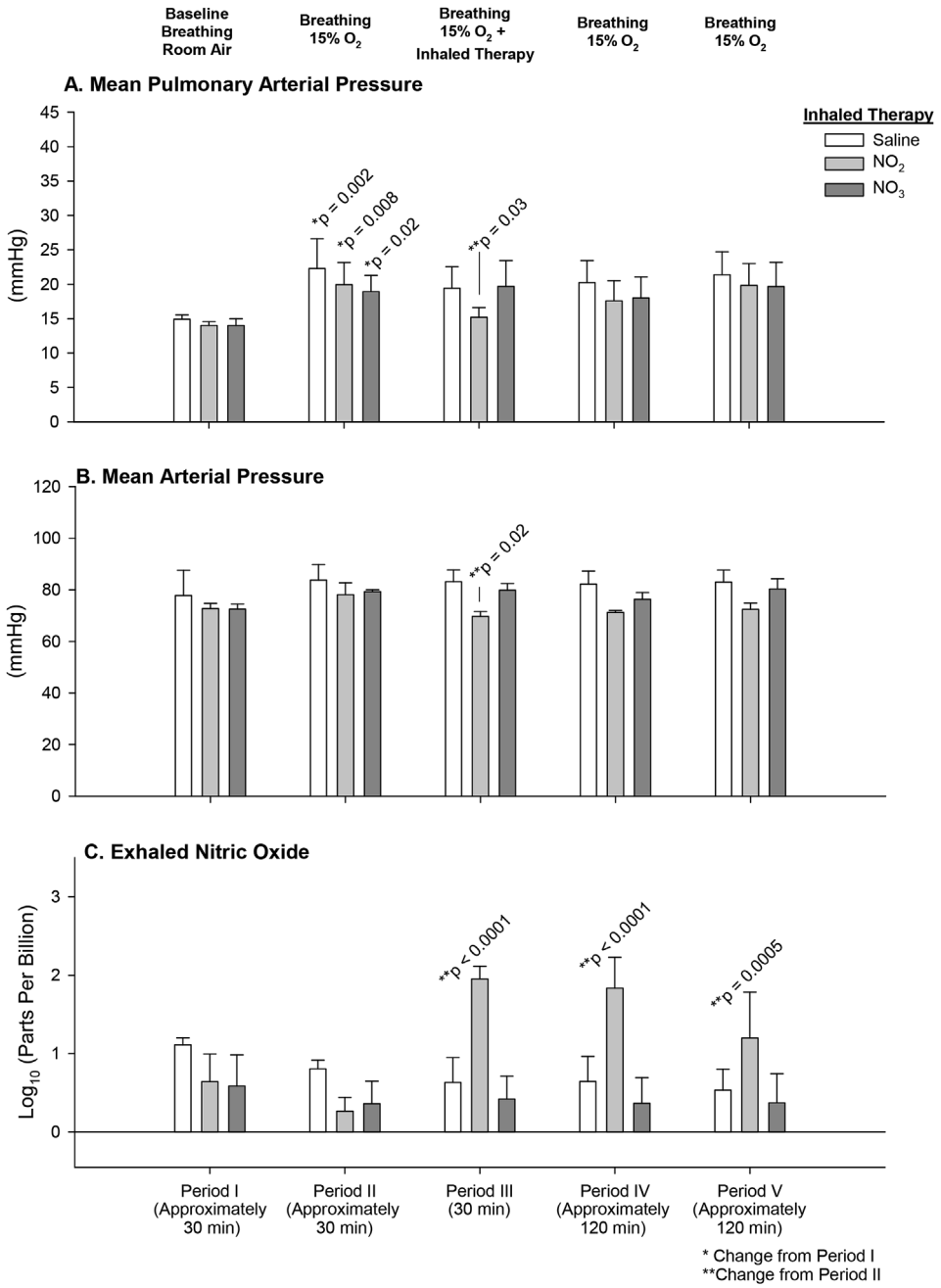

Fig. 2. Serial mean ± SE values for effects of inhaled study drugs on vascular tone and exhaled nitric oxide production. The study is divided on the x-axis into five serial time periods I (baseline), II (initiation of hypoxia) III, (inhalation treatment) IV, (acute effects after terminating inhalation treatment) V, (persistent effects after terminating inhalation treatment). The mean values during each period are shown on the y-axis by different shaded vertical bars (open, saline; light grey, nitrite; dark grey, nitrate) for mPAP (Panel A), MAP (Panel B) and log 10 exhaled NO levels (Panel C). For unlogged NO values please see supplementary e-Fig. 2.

show evidence that this dose of inhaled nitrite, but not of inhaled nitrate or saline, can lower mPAPs and MAPs, which is associated with elevations in exhaled NO. Changes in the pulmonary vascular resistance index (PVRI) followed the same pattern as mean mPAPs but were not statistically significant, likely due to a power issue with increased variability in this measure and only three animals studied per group (Table 1). During nitrite inhalation, the systemic vascular resistance index (SVRI) significantly decreased in comparison to the period of hypoxia immediately before starting the inhaled therapy (Table 1). There were no significant changes observed in mean SVRI for animals receiving inhaled saline or inhaled nitrate during this same time period. During the last time period studied (Period V) there were late significant increases in mean SVRI for animals receiving inhaled saline or inhaled nitrate compared to this same time period not seen in animals receiving nitrite. Potentially a late effect of nitrite may have prevented such late increases in mean SVRI seen in the other two study arms.

3.3. Randomized controlled trial: summary of blood nitrate and nitrite levels

Serial mean arterial and venous nitrite and nitrate levels for each of the three treatments were analyzed for the five periods (Fig. 3; see Supplementary Fig. 3 for non-logarithmic data). The animals receiving inhaled nitrite therapy had a significant rise in arterial and venous mean nitrite and nitrate levels during and for several hours after inhalation ($p = 0.02$ to $p < 0.0001$). The animals receiving inhaled

Table 1
Serial mean ± SE values for effects of inhaled study drugs on additional hemodynamic and laboratory parameters. The study is divided into five serial time periods similarly as for Fig. 1. The mean values and SE for each study drug are shown across all five periods for pH, arterial partial pressure of oxygen (PaO2), arterial partial pressure of Carbon Dioxide (PCO2), bicarbonate (HCO3), Cardiac Index (CI), pulmonary vascular resistance index (PVRI), systemic vascular resistance index (SVRI), hemoglobin and hematocrit.

Treatment	Period I (Approximately 30 min)	Period II (Approximately 30 min)	Period III (30 min)	Period IV (Approximately 120 min)	Period V (Approximately 120 min)	
	pH					**Arterial Blood Gas**
Saline	7.36 ± 0.05	7.31 ± 0.01^{*a}	7.34 ± 0.04	7.34 ± 0.01^{*c}	7.33 ± 0.01	
Nitrite	7.37 ± 0.02	7.31 ± 0.01^{**a}	7.28 ± 0.03	7.32 ± 0.01	7.31 ± 0.03	
Nitrate	7.32 ± 0.02	7.31 ± 0.02	7.30 ± 0.01	7.32 ± 0.01	7.31 ± 0.02	
	PaO_2 (mmHg)					
Saline	136.5 ± 12.8	77.9 ± 4.2^{***a}	63.4 ± 16.8	72.8 ± 2.7	66.7 ± 7.0	
Nitrite	127.0 ± 34.7	70.4 ± 11.6^{***a}	76.3 ± 14.8	67.4 ± 5.5	70.6 ± 4.7	
Nitrate	111.5 ± 10.6	71.1 ± 8.0^{**a}	85.6 ± 23.7	86.6 ± 10.4	70.5 ± 5.4	
	pCO2 (mmHg)					
Saline	34.2 ± 5.69	37.9 ± 4.74	35.9 ± 5.75	34.6 ± 2.64^{*c}	38.9 ± 3.90	
Nitrite	32.6 ± 2.45	37.6 ± 4.94^{*a}	42.4 ± 4.03^{*c}	35.1 ± 2.27	36.9 ± 2.31	
Nitrate	34.0 ± 4.73	35.5 ± 4.29	35.7 ± 5.18	32.8 ± 2.72	33.6 ± 2.48^{*d}	
	HCO3 (mmol/L)					
Saline	19.1 ± 1.96	19.2 ± 2.02	19.2 ± 2.11	18.9 ± 1.28	20.9 ± 0.90	
Nitrite	17.6 ± 3.35	19.3 ± 2.92^{***a}	19.8 ± 2.66	18.3 ± 1.53^{**c}	19.0 ± 2.09^{**d}	
Nitrate	17.9 ± 2.75	17.8 ± 2.57	17.7 ± 2.85	16.9 ± 1.56^{*c}	17.5 ± 1.79^{**d}	
	Cardiac Index (L/min/kg)					**Hemodynamic**
Saline	0.15 ± 0.05	0.10 ± 0.05	0.10 ± 0.05	0.11 ± 0.03	0.15 ± 0.04	
Nitrite	0.13 ± 0.03	0.12 ± 0.05	0.11 ± 0.05	0.15 ± 0.02	0.13 ± 0.02	
Nitrate	0.13 ± 0.03	0.13 ± 0.05	0.11 ± 0.04	0.15 ± 0.02	0.11 ± 0.04	
	Pulmonary Vascular Resistance Index (dynes/cm^5/kg)					
Saline	4345 ± 1280	4982 ± 124	6481 ± 512	7333 ± 1220	7179 ± 1382	
Nitrite	5165 ± 1509	6226 ± 793	3376 ± 370	6426 ± 1058	8767 ± 1016	
Nitrate	3214 ± 564	4587 ± 1806	6779 ± 1815	6792 ± 1161	7314 ± 841	
	Systemic Vascular Resistance Index (dynes/cm^5/kg)					
Saline	43425 ± 10679	34748 ± 7846	39484 ± 7014	40905 ± 2313	48427 ± 9523^{**d}	
Nitrite	46663 ± 10336	39902 ± 9586	29005 ± 3201^{*b}	40260 ± 4750	39528 ± 3627	
Nitrate	45326 ± 11933	36388 ± 5439	40653 ± 5976	43223 ± 5038	47936 ± 6988^{**d}	
	Hemoglobin (g/dL)					**Hematology**
Saline	12.9 ± 1.78	13.0 ± 1.24	13.4 ± 1.13	12.7 ± 0.67	11.5 ± 0.70	
Nitrite	11.5 ± 0.93	12.6 ± 0.92^{**a}	12.4 ± 0.58	12.0 ± 0.41	11.1 ± 0.82^{**d}	
Nitrate	11.9 ± 0.64	12.2 ± 0.42	12.2 ± 0.22	12.1 ± 0.07	12.2 ± 0.49	
	Hematocrit (%)					
Saline	38.7 ± 5.55	39.0 ± 3.46	40.0 ± 3.46	38.0 ± 2.05	34.5 ± 1.50	
Nitrite	34.7 ± 2.96	38.0 ± 2.89^{**a}	37.3 ± 1.76	35.8 ± 1.38^{*c}	33.5 ± 2.47^{**d}	
Nitrate	36.0 ± 2.08	36.7 ± 1.20	36.3 ± 0.67	36.3 ± 0.21	36.5 ± 1.55	

P-Values.
*0.01–0.05.
** 0.001–0.01.
*** ≤ 0.001.
Periods Compared.
[a] II to I.
[b] III to II.
[c] IV to II.
[d] V to II.

nitrate treatment had a significant rise in arterial and venous mean nitrate levels during and for several hours after inhalation ($p = 0.001$ to $p < 0.0001$) but no significant elevations in arterial or venous mean nitrite levels throughout the whole experiment duration (all, $p > 0.05$). The inhaled saline-treated animals had no consistent significant elevations in arterial or venous mean nitrate or nitrite levels throughout. These data suggest that nitrite delivered to the respiratory system can be converted to nitrate intrapulmonary or intravascularly, while inhaled nitrate, under these conditions and at the tested dose, is not reduced to nitrite in a significantly measurable way.

3.4. Randomized controlled trial: other laboratory parameters

In animals receiving inhaled nitrite, saline and nitrate, the pattern of changes in mean hemoglobin and hematocrit levels were similar throughout. There was an increase in mean hemoglobin and hematocrit levels when the inspired oxygen concentration was lowered to 15% seen in all three treatment group that only reached statistical significance in the nitrite group (Table 1). These increases were transitory and, by the last period of the experiment, while the animals were still breathing 15% oxygen and more than 2 h after the inhaled treatment had ended, hemoglobin and hematocrit levels had return to levels similar to baseline. The decrease in mean hemoglobin and hematocrit levels as one would expect at the end of the experiment reached significance only with nitrite inhalation (Table 1).

Lowering the inspired oxygen concentration from 21% to 15% resulted in decreases in pH across all treatment group, albeit only statistically significant for animals receiving nitrite and animals receiving saline. Arterial partial pressure of Carbon Dioxide (PCO2) levels increased during this same initial period of hypoxia, as well as bicarbonate (HCO3) levels. These responses we suspect were related to the effects of an increase in sedation to tolerate hypoxia. These mean increases were only statistically significant for the group of animals receiving inhaled nitrite. Interestingly, we observed a further significant increase in PCO2 levels during the inhaled therapy period only for animals receiving nitrite, while these levels remained unchanged

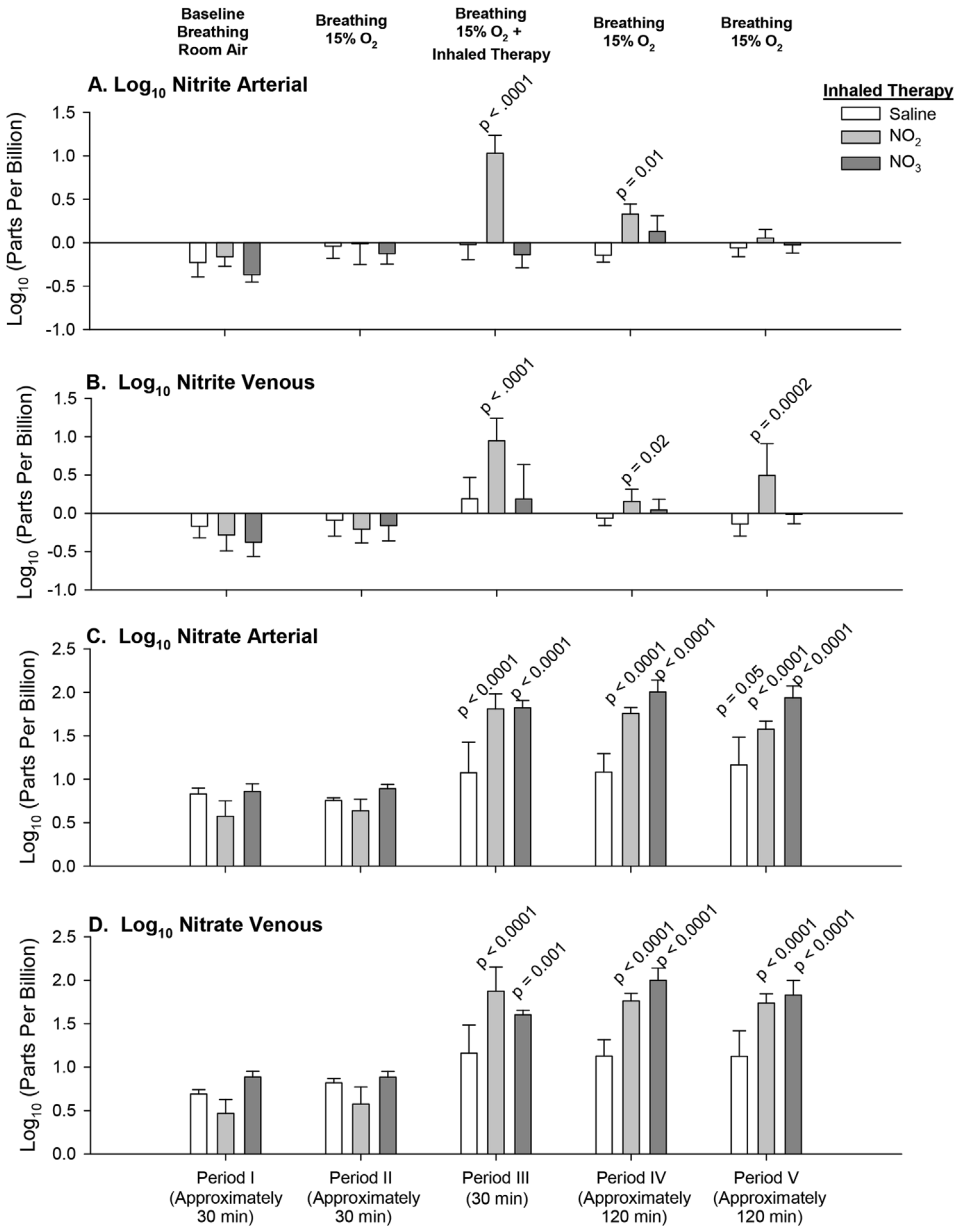

Fig. 3. Serial mean ± SE values in the arterial and venous beds of inhaled study drugs. The format is similar to Fig. 2 but now log[10] nitrite and nitrate arterial and venous blood values are plotted instead of vascular pressures and exhaled NO levels. For unlogged nitrite and nitrate values please see supplementary e-Fig. 3.

during inhaled therapy with saline or nitrate. Another potential unexplained late effect of nitrite akin to the late changes in mean SVRI.

3.5. Dose-finding study

In the dose-finding study, three of the nine animals received inhaled nitrate at increasing doses. At each dose studied in each animal by visual inspection (Fig. 4; see Supplementary Fig. 4 for non-logarithmic exhaled NO data) and averaging over all doses given and all animals studied, there was no significant change in the experimental pulmonary hypertension produced with hypoxia and U46619 (mimetic of thromboxane A_2) (overall mean change ± standard error in elevated pulmonary pressures from hypoxia with inhaled nitrate therapy averaging over all inhaled nitrate doses employed and animals studied, 0.67 ± 0.86 mmHg, p = 0.44). In Fig. 3, the effects of increasing individual inhaled nitrate dose are shown in two animals, one animal receiving hypoxia alone (Animal 1) and one hypoxia plus U46619 (Animal 2). Serial measures are shown of mPAP (top panels), MAP (middle panels) and exhaled NO level responses (bottom panels) while breathing room air and after reducing oxygen to 15% followed by increasing inhaled nitrate doses (from 30 to 300 mg/min over 120 min). Reducing oxygen from room air to 15% raised mPAP with or without U46619. However, inhaled nitrate did not, at any dose given (including 300 mg/min), have any marked effect on mPAP, MAP or exhaled NO. These data suggest these larger doses of inhaled nitrate than those studied in the randomized controlled trial (30 mg/min) also do not result in the reduction of nitrate to nitrite to NO and/or lowering of mPAP up to approximately 20 h after the nitrate inhalation ended. MAP differences throughout the experiment were not large enough to make inferences from the individual data for the two animals.

In the dose-finding study, three of the nine animals received inhaled saline at increasing doses. At each dose studied in each animal by visual inspection and averaging over all doses given and all animals studied, there was no change in the experimental pulmonary hypertension produced with hypoxia and U46619 (overall mean change ± standard error in elevated pulmonary pressures from hypoxia with inhaled saline

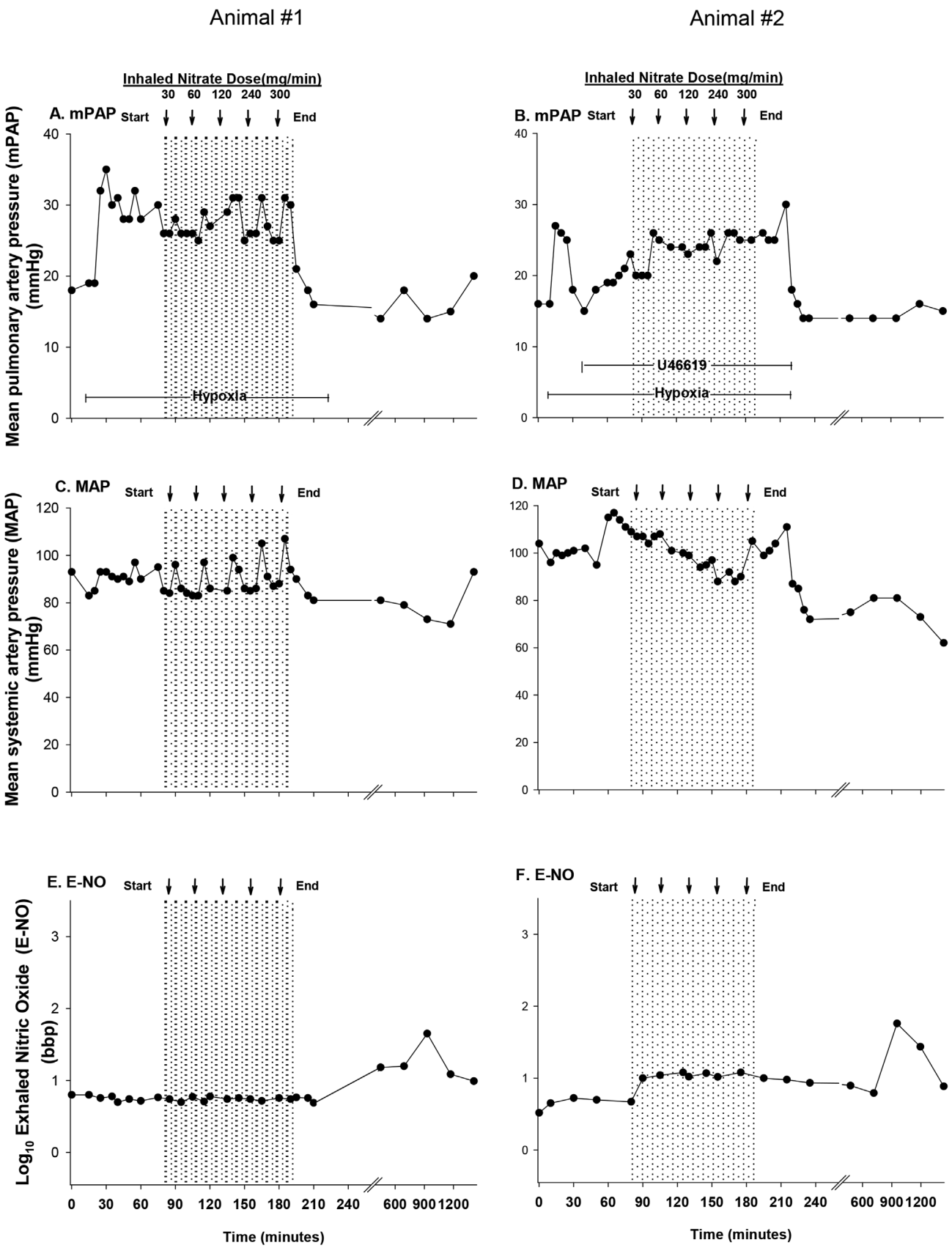

Fig. 4. Illustrative examples of effects of increasing doses of inhaled nitrate on vascular tone and exhaled nitric oxide production. The format is similar to Fig. 1 except now increasing doses of inhaled nitrate 30–300 mg/min are shown for two animals. For unlogged NO please see supplementary e-Fig. 3.

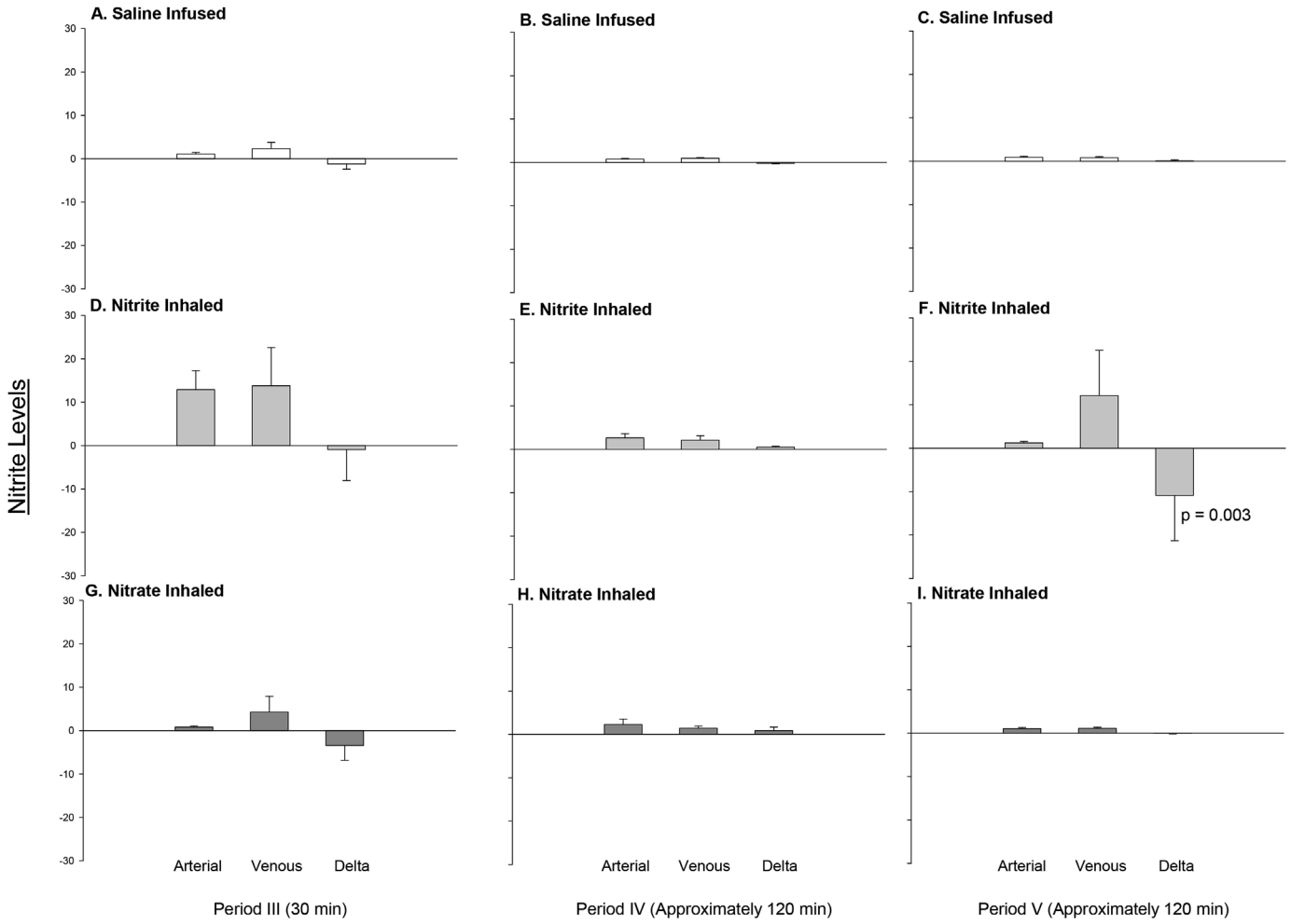

Fig. 5. After inhalation of inhaled study drugs serial mean ± SE values in arterial and venous blood of nitrite levels and arterial-venous nitrite level differences. The format is similar to Fig. 2 but only time period III, IV, and, V are shown and now arterial and venous blood values for nitrite and the arterial venous nitrite differences are plotted instead of vascular pressures and exhaled NO levels.

therapy averaging over all inhaled saline doses employed and animals studied, 0.04 ± 0.75 mmHg, p = 0.96).

In the dose-finding study, three of the nine animals received inhaled nitrite at increasing doses. At most doses studied in each animal by visual inspection and averaging over all doses given and all animals studied, there was a significant change in the experimental pulmonary hypertension produced with hypoxia and U46619 (overall mean change ± standard error in elevated pulmonary pressures from hypoxia with inhaled nitrite therapy averaging over all inhaled nitrate doses employed and animals studied, −2.90 ± 1.05 mmHg, p = 0.007).

3.6. Randomized controlled trial: summary of arterial to venous differences in nitrite and nitrate levels

In terms of nitrite levels, animals receiving inhaled nitrite had a significant mean difference in arterial to venous nitrite levels late 2–4 h after inhalation treatment (p = 0.003) (Fig. 5). This represented a late increase in mean venous but not arterial levels of nitrite. In all other animals receiving saline, nitrite and nitrate inhaled infusions, there were no other significant mean differences in arterial verses venous levels of nitrite throughout the study (all, p > 0.05).

In terms of nitrate levels, the animals receiving saline inhaled infusions had no significant mean differences in arterial and venous nitrite levels throughout the study (all, p > 0.05) (Fig. 6). The animals receiving inhaled nitrite therapy had a significant mean difference in arterial and venous nitrate levels during inhalation period (p = 0.01). This represented an increase in the mean venous levels of nitrates greater than arterial levels. There were no other significant arterial-venous mean differences in mean nitrate levels in this group throughout the study (all, p > 0.05) but notably with inhaled nitrite infusions, the mean venous levels of nitrate were always nominally higher than the arterial levels of nitrate. The animals receiving nitrate therapy had a significant mean difference in arterial and venous levels (p = 0.03) during the inhalation period. This represented an increase in the mean arterial levels of nitrate greater than venous levels. There were no other significant arterial-venous mean differences in mean nitrate levels in this group throughout the study (all, p > 0.05).

3.7. Western blotting

Fig. 7 shows the presence of xanthine oxidoreductase (XOR) in canine lung and liver in comparison to rat liver and lungs. Levels in canine liver are similar to those we have previously shown in rat liver [32]. As seen in Fig. 7, both rat and canine lung tissue contained substantial amount of XOR enzyme, while liver serves as a positive control in both animal species.

4. Discussion

We hypothesized that the reduction of nitrate to nitrite and then further reduction of nitrite to NO would occur locally in the lung,

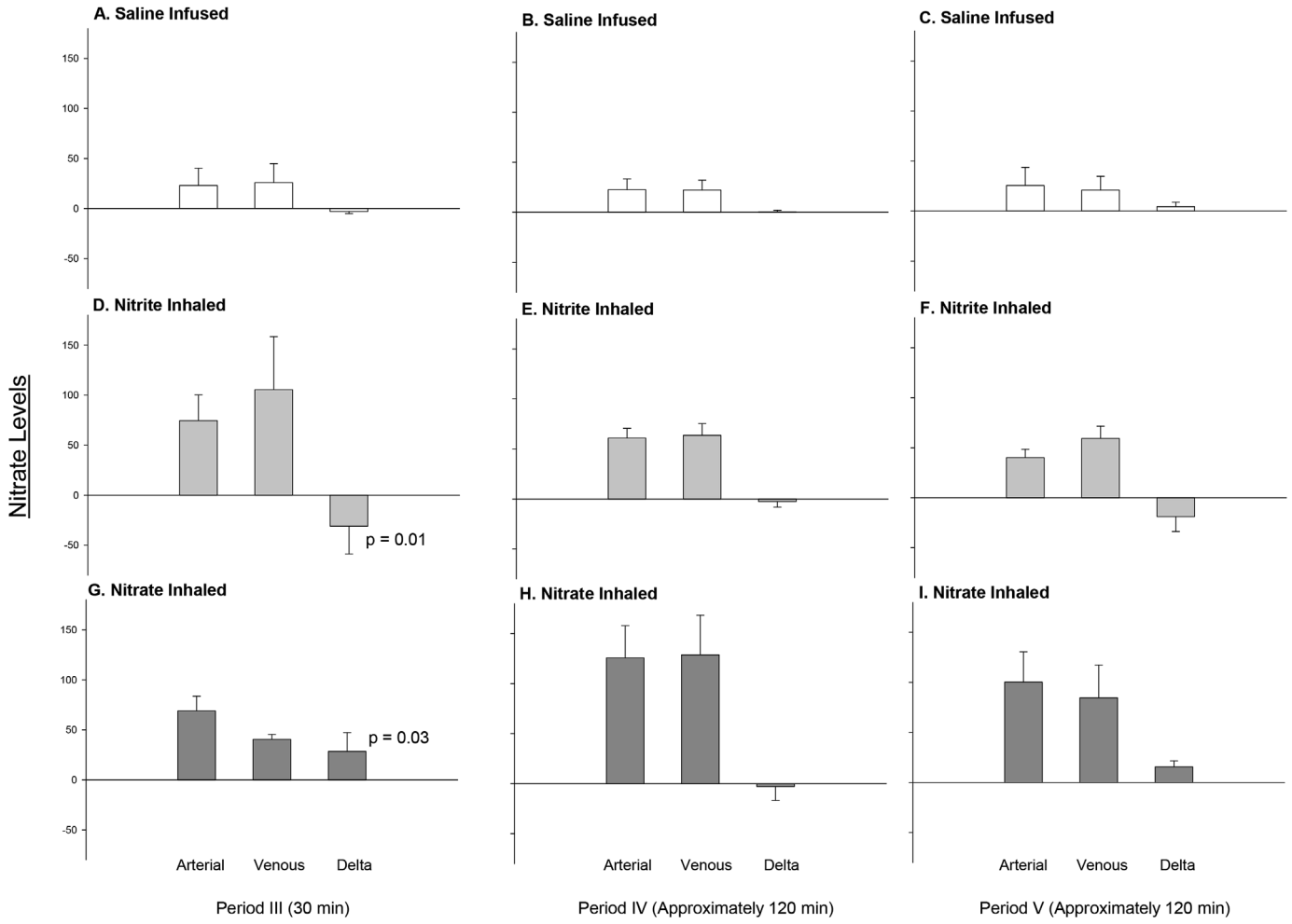

Fig. 6. After inhalation of inhaled study drugs serial mean ± SE values in arterial and venous blood of nitrate levels and arterial-venous nitrate level differences. The format is similar to Fig. 2 but now only time period III, IV, and, V are shown and arterial and venous blood values for nitrate and the arterial venous nitrate level differences are plotted instead of vascular pressures and exhaled NO levels.

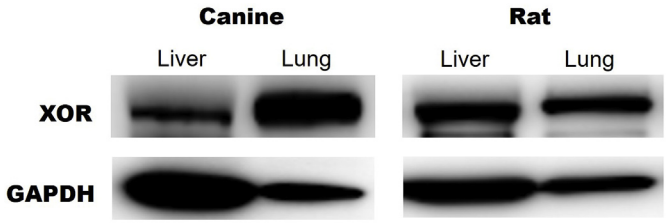

Fig. 7. Western Blots. Lung and liver obtained from normal animals showing presence of xanthine oxidoreductase (XOR).

mediated by non-specific nitrate/nitrite reductases present in pulmonary tissue. If this pathway is able to generate physiologically relevant amounts of NO locally and induce vascular effects, nitrate could be considered as a feasible alternative to inhaled NO or inhaled nitrite for lowering pulmonary arterial pressures, with the advantages of having a longer half-life and non-reactivity with hemoglobin. To test this hypothesis, we first measured and confirmed the expression of XOR, an endogenous enzyme with known nitrate reductase activity, in canine lung tissue. Next, we conducted a trial of inhaled nitrate in a canine model of hypoxic-induced acute pulmonary hypertension using a negative control arm, inhaled saline, and a positive control arm, inhaled nitrite, known to have vascular effects in animal models of acute pulmonary hypertension [40,41]. NO generation in the lungs was monitored by continued measurement of exhaled NO levels. The potential vascular effects of NO generation were monitored through continuous hemodynamic monitoring of pulmonary and systemic arterial pressures. In addition, arterial and venous blood levels of nitrate and nitrite were measured at pre-defined timepoints to document systemic absorption of the inhaled therapies and as surrogates of the underlying chemical reactions taking place. Finally, we conducted additional dose-response experiments using increasing doses of inhaled nitrate as well as long observation times to control for the possibility of either the nitrate dose not being high enough or the serial reduction to NO occurring slowly and the effects appearing delayed in time.

Hypoxia effectively induced acute pulmonary artery hypertension in canines. This was rapidly and effectively reversed by the administration of inhaled nitrite but not by inhaled nitrate at the tested doses or inhaled saline. Nitrite inhalation resulted in reversal of pulmonary hypertension through its reduction to NO, as confirmed by the statistically significant elevations in mean exhaled NO levels during and up to 4 h following its administration. In contrast, treatment with inhaled nitrate or inhaled saline did not result in significant elevations in exhaled NO, both during inhalation or after treatment discontinuation. During nitrite inhalation, nitrite systemic blood levels increased alike in the arterial and venous system. Nitrite administered by inhalation reaches the left heart and systemic arterial circulation before it reaches the systemic venous beds, so nitrite levels in the arterial side could be theoretically expected to be higher than in venous side. The reason why we found the opposite pattern is that nitrite is easily oxidized into nitrate by oxygenated hemoglobin, the concentration of which is higher on arterial side.

In addition to the lowering of pulmonary arterial pressures, nitrite inhalation also resulted in a significant decrease of systemic MAP. After completion of the 30-min nitrite inhalation period, hypoxia-induced pulmonary hypertension rapidly recurred and the MAP returned to normal. In summary, inhaled nitrite, our hypothesized positive control, was confirmed to be reduced to NO in the canine lung tissue, locally increasing exhaled NO levels and decreasing pulmonary arterial pressure; as well as being absorbed into the systemic circulation, increasing systemic arterial and venous blood levels of nitrite and exerting systemic vascular effects.

There were some unexpected findings associated to inhalation of nebulized nitrite. We found that nitrite inhalation resulted in prolonged increases in exhaled NO levels which persisted for more than 4 h after the inhalation of nitrite ceased, despite the measurable vascular effects being limited to the inhalation period. Arterial levels of nitrite became negligible 2 h after nitrite inhalation was stopped. In contrast, nitrite venous levels persisted elevated throughout a 4-h period after inhalation ceased. Interestingly, arterial and venous nitrate levels were elevated during this 4-h period as well, and there was a late surge of venous, but not arterial, nitrite levels occurring more than 4 h after the end of the inhalation period. This delayed increase in venous nitrite levels occurred concurrently with an increase of nitrate levels in venous more than in arterial blood. This is consistent with the hypothesis that, during inhalation, nitrite reaches the systemic circulation and is subsequently stored in tissues, potentially in muscle tissue. Nitrite stored in tissues can be then oxidized to nitrate by agents such as oxygenated myoglobin [62], slowly releasing nitrate into the systemic circulation. This can explain why nitrate levels remained elevated in both arterial and venous blood for hours after inhalation of nitrite ceased. Additionally, nitrite can be converted to NO by local nitrite reductases, such as XOR, present in myocytes and other tissues, and released from these storage tissues at low concentrations, not sufficient to elicit prolonged measurable vascular effects but resulting in late increases in venous nitrite level. Further study is needed to reproduce and investigate the cause of this late surge of venous nitrite and venous nitrate levels occurring hours after nitrite inhalation. However, prolonged effects of nitrite infusions after cessation of delivery have been noted with intravenous nitrite [63] and increases in exhaled NO up to 20 min beyond the period of nitrite inhalation has been previously shown in newborn lambs [40]. Our findings raise not only the possibility of using this delayed release of nitrite from tissue and conversion to NO therapeutically, but also concerns for potential delayed toxic side effects when using inhaled nitrite as a therapeutic option.

As expected, inhalation of nebulized saline, our negative control, did not reverse hypoxia-induced hypertension, lower MAP, increased exhaled NO levels, raise venous or arterial nitrite or nitrate levels or have differential effects on these levels on arterial *versus* venous vascular beds. This confirms that, in response to non-specific inhalation therapy, exhaled NO is not released. Unexpectedly, our test drug (inhaled nebulized nitrate) had effects more similar to our negative control (inhaled saline) than to our positive control (inhaled nitrite). In response to nitrate inhalation, its levels increased more in the arterial than venous blood systems, as expected, confirming that nitrate was absorbed into the bloodstream from the lungs. The greater nitrate levels found in the arterial phase are expected during the inhalation therapy, since nitrate absorbed into bloodstream is first transported into the left heart and systemic arterial circulation before it reaches systemic veins. Importantly, there were no measurable increases in arterial or venous nitrite levels associated with these increases in arterial and venous nitrate levels, despite our confirmation of the expression of a known endogenous nitrate reductase, XOR, in the lung of the study animals as well as in a small animal model. This, along with no measurable increases in levels of exhaled NO after inhalation of nitrate, is strong chemical evidence that the reduction of nitrate to nitrite does not take place in a measurable way at the used dose of inhaled nitrite (30 mg/min). Importantly, there were no vascular effects of nitrate inhalation on hypoxia-induced pulmonary hypertension or changes in systemic blood pressures, documenting there are no physiological effects of inhaled nitrate at this dose. In the attempt to establish possible dose-dependence, we increased doses of inhaled nitrate from 30 to 300 mg/min and observation times up to 20 h and found no chemical or physiological evidence that nitrate was converted to nitrite or NO in canine lungs. Thus, we concluded that under the conditions seen in our experimental hypoxia-induced pulmonary hypertension model, inhaled nitrate is not converted to nitrite by nitrate reductases in a measurable way in the canine lung.

Dysfunction within the NO signaling pathway is known to be an important contributor to the development of pulmonary hypertension in mammals [64,65]. Although this dysfunction has not been fully elucidated, it appears to involve both decreased NO production [66] as well as NO resistance [2,67,68]. Recent insight on the alternative pathway for NO generation involving the serial reduction of nitrate and nitrite to NO [14,16] has generated interest in inhaled nitrite as a potential alternative to inhaled NO for pulmonary hypertension. Since the initial discovery of the nitrite reductase activity of deoxygenated hemoglobin [17], inhaled nitrite has been investigated as a selective pulmonary vasodilator in newborn sheep [40,41], and systemic administration of nitrite was shown to reverse pulmonary hypertension in rodents [42] and canines [38] as well as hypoxia-induced pulmonary vasoconstriction in healthy humans [69]. However, intravenous administration of nitrite can lead to undesired systemic vascular effects as well as rapid elevations of met-hemoglobin. In addition, intravenous nitrite administration can only be done safely in a hospital setting and is not feasible as a long-term therapy. Due to these limitations, inhaled nebulized nitrite has been recently evaluated for pulmonary hypertension-related complications in humans [45,70]. Our findings confirm that inhaled nebulized nitrite is effective at lowering pulmonary pressures through its reduction to NO in the lung tissue in a large animal model of acute pulmonary hypertension.

While the use of inhaled nitrite as a potential therapy for pulmonary hypertension has been the focus of a large body of research in the past decade, the possibility of using targeted nitrate therapy for pulmonary hypertension has not been yet explored. The reduction of nitrate to nitrite *in vivo* has largely been thought of as dependent of commensal bacteria in the enterosalivary pathway. As such, previous research and ongoing clinical trials have focused on dietary nitrate interventions for cardiovascular therapeutics, including pulmonary hypertension [39,54,55,71]. However, recent studies have shown that nitrate can be reduced to nitrite *in vitro* and *in vivo* in several organs through non-specific enzymatic pathways [30–35]. Jansson et al. demonstrated nitrate reduction to nitrite *in vivo* in germ-free mice, confirming that this step of the nitrate-nitrite-NO pathway can take place in the absence of specific bacterial nitrate reductases [30]. In addition, nitrate reduction to nitrite has been demonstrated *in vitro* in rodent tissue from gastrointestinal tract [30], liver [30,34], muscle [31,32,34] and heart [30,33], as well as in human liver tissue [30]. Most of these studies have suggested that the mammalian reduction of nitrate to nitrite is mostly mediated by a family of enzymes that use molybdenum as a cofactor and are structurally very similar to bacterial nitrate reductases [72]. In mammals, this family comprises XOR and AO [72]. Previous studies have shown that inhibition of XOR and/or AO with specific pharmacologic inhibitors substantially decreases the reduction of nitrate to nitrite in mammalian tissues [30–33]. Although nitrate reduction to nitrite has been consistently demonstrated in gastrointestinal tissue, liver and other organs; little is known about this pathway in the lung. It has been shown that nitrate can in fact be reduced to nitrite lung tissue, although to a lesser extent in comparison to other tissues [30]. However, the underlying pathway and whether it may be stimulated during disease states is not well understood. The potential advantages of using inhaled nitrate instead of inhaled nitrite or NO are the longer half-life of nitrate (~5–6 h) [56] and slower reduction and its non-reactivity with hemoglobin, thus decreasing the risk of increased

methemoglobin. To our knowledge, this is the first study evaluating inhaled nebulized nitrate as a potential precursor of NO in the lung tissue. We confirmed the presence of a known nitrate reductase enzyme, XOR, in the lungs of the animals involved in these experiments. However, nitrate inhaled therapy at the tested doses failed to generate local increases in NO, as shown by the lack of increase in exhaled NO levels, or physiologic vascular effects on pulmonary pressures.

Our study has several limitations. The hypoxia-induced acute pulmonary hypertension model and/or animal species may not be relevant to the underlying complex pathophysiology of pulmonary hypertension patients. In addition, some missing cofactor may be necessary for this reaction to take place and/or a higher dose or length of observation or level of deoxygenation could be necessary. Importantly, it has been shown in humans that lower levels of XOR are present in healthy lungs in comparison to other organs; and that the lung expression of XOR increase in disease states, and especially during hypoxia [2,73,74]. It is possible that higher levels of XOR are required for nitrate reduction to be significant. Pulmonary hypertension patients with varying degrees of chronic hypoxia may have chronic increases in XOR expression that our acute model of pulmonary hypertension may not be able to reproduce. Pulmonary nitrate reduction may be dependent on enzymatic pathways other than XOR in humans, and these unknown pathways may not be present in canines. Interestingly, in a previous study that was able to show the reduction of nitrate to nitrite *in vitro* in lung tissue, the use of an XOR inhibitor, oxypurinol, did not inhibit this conversion, suggesting that other enzymatic processes may be involved [30]. It is possible that, even if XOR does not reduce nitrate efficiently in the lung itself, inhaled nitrate could have passed into the bloodstream, be reduced into nitrite and then XOR in the lung could have reduced nitrite further to NO, potentially reversing the acute pulmonary hypertension. However, we believe that this possibility would have been accompanied by systemic vascular effects, instead of selective pulmonary vascular effects, which makes it somehow less appealing from the therapeutic point of view. Lastly, our study did not evaluate the effects of the different interventions on methemoglobin levels.

In summary, our data confirms that inhaled nebulized nitrite effectively reverses acute hypoxia-induced pulmonary hypertension in canines. This effect is at least in part mediated by nitrite reduction to NO in the lung tissue and/or in the vasculature. These findings agree with previous research using inhaled, dietary or intravenous nitrite in animal models and humans [38–45]. Interestingly, despite the vascular effects of nitrite being limited to the inhalation period, we found an unexpected prolonged increase of exhaled NO as well as a late surge in systemic blood levels of venous nitrite and venous and arterial nitrate, suggesting the existence of a pathway involving tissue storage and late recirculation of nitrite. This finding, if confirmed, may challenge the prevailing notion that the effects of inhaled nitrite therapy—similarly to inhaled NO—are limited to the inhalation period. The potential for more prolonged effects of nitrite therapy associated to nitrite tissue storage, local oxidation and/or reduction reactions and recirculation of nitrite to the vasculature merit further research efforts. Nitrite-induced S-nitrosylation, recently studied in a rat model of hypoxic pulmonary hypertension, could also have a role in the observed effects of nitrite [42]. Still, in order to implement this therapy, there is an array of questions that needs to be addressed, including whether there is a specific dose and/or formulation of nitrite that minimally affects systemic blood pressure and that minimizes the development of methemoglobin. Ideally, a hand-held device that could be used multiple times a day with effects lasting several hours and a safe side effect profile represents a potentially practical, biologically plausible approach to investigate for diseases requiring prolonged NO therapy. Our study did not find any significant effects of inhaled nitrate on measured chemical or physiological parameters. The lack of effects of inhaled nitrate in this canine model of acute hypoxia-induced pulmonary hypertension is surprising and leads us to consider other ways of employing nitrate to effectively utilize the nitrate/nitrite reductases expressed in tissues to lead to NO formation.

Author contributions

ICP designed the study, performed experiments, provided data analysis and figure generation, analyzed and interpreted the data and wrote/edited the manuscript; JS performed all statistical analysis; SBS, JF, JWP and CG performed relevant experiments; CN, BP and ANS designed the study, analyzed and interpreted the data, and wrote/edited the manuscript.

All authors reviewed and approved the final version of the manuscript.

Conflicts of interest

Alan N. Schechter is listed as a co-inventor on several patents issued to the National Institutes of Health for the use of nitrite salts for the treatment of cardiovascular diseases. He receives royalties based on NIH licensing of these patents for clinical development but no other compensation. All other authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.07.001.

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