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A pilot study on the kinetics of metabolites and microvascular cutaneous effects of nitric oxide inhalation in healthy volunteers

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Abstract

Rationale

Inhaled nitric oxide (NO) exerts a variety of effects through metabolites and these play an important role in regulation of hemodynamics in the body. A detailed investigation into the generation of these metabolites has been overlooked.

Objectives

We investigated the kinetics of nitrite and S-nitrosothiol-hemoglobin (SNO-Hb) in plasma derived from inhaled NO subjects and how this modifies the cutaneous microvascular response.

Findings

We enrolled 15 healthy volunteers. Plasma nitrite levels at baseline and during NO inhalation (15 minutes at 40 ppm) were 102 (86–118) and 114 (87–129) nM, respectively. The nitrite peak occurred at 5 minutes of discontinuing NO (131 (104–170) nM). Plasma nitrate levels were not significantly different during the study. SNO-Hb molar ratio levels at baseline and during NO inhalation were 4.7E-3 (2.5E-3–5.8E-3) and 7.8E-3 (4.1E-3-13.0E-3), respectively. Levels of SNO-Hb continued to climb up to the last study time point (30 min: 10.6E-3 (5.3E-3-15.5E-3)). The response to acetylcholine iontophoresis both before and during NO inhalation was inversely associated with the SNO-Hb level (r: -0.57, p = 0.03, and r: -0.54, p = 0.04, respectively).

Conclusions

Both nitrite and SNO-Hb increase during NO inhalation. Nitrite increases first, followed by a more sustained increase in Hb-SNO. Nitrite and Hb-SNO could be a mobile reservoir of NO with potential implications on the systemic microvasculature.

Introduction

Over the past years the role of NO in biology has increased tremendously and is involved in diverse functions such as bacterial defense, neurotransmission and reproduction [1–4]. NO is a highly diffusible gas synthesized by a group of nitric oxide synthases (NOS) [5]. It was identified in 1980s and initially referred as endothelium-dependent relaxation factor [6–8]. Three isoforms of NOS exist but the endothelial NOS (type 3 NOS) generates NO in the pulmonary vascular bed [9]. Upon generation, NO diffuses to adjacent cells [10]. NO's half-life can be as low as 1.8 ms in the presence of mM concentrations of hemoglobin (Hb) [8, 11, 12], and therefore it is likely not the principal effector molecule for many NO bioactivities. NO can be rapidly oxidized to nitrate (NO_3^-) and nitrite (NO_2^-) [13], or taken by hemoglobin (Hb) to form nitrosyl-Hb (Hb:[Fe]NO) or S-nitrosothiol-Hb (SNO-Hb) [14].

NO administered via inhalation relaxes the pulmonary vasculature. Inhaled NO in critically ill neonates with pulmonary hypertension improves oxygenation [15, 16] and is currently approved by the US Food and Drug Administration [17, 18] for treatment of term and near-term neonates with hypoxic respiratory failure associated with clinical or echocardiographic evidence of pulmonary hypertension. Other studies have suggested that inhaled NO could be used to treat a wide spectrum of cardiopulmonary conditions, including acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), acute pulmonary embolism (PE), hypoxemic respiratory failure and pulmonary hypertension [19–23].

Inhaled, NO diffuses across the lung, reaching the smooth muscle cells of subjacent vessels where it causes selective pulmonary vasodilation [24, 25]. Besides local pulmonary effects, mounting evidence supports that inhaled NO exerts a variety of systemic effects. A number of studies have demonstrated that inhaled NO can affect multiple organs [26–35]. Indeed inhaled NO can be protective in brain injury as demonstrated by the hypoxia-ischemia or traumatic brain injury models [30–35]. Given the efficient scavenging by hemoglobin, the systemic effects are unlikely caused by direct effects of the inhaled NO itself [5]. Nitrite, low molecular weight SNO (eg. S-Nitrosoglutathione (GSNO)) or protein bound SNO species, may be involved in transporting the NO signal to the systemic circulation [36]. In fact, during hypoxic conditions, nitrite can regenerate NO by nitrite reductases [37, 38] and SNO-Hb is bioactivated by transfering nitrosonium (NO^+) to other thiols [14, 39].

Controversy persists on whether both nitrite in plasma and SNO-Hb in erythrocytes increase during NO inhalation [40]. In fact, there remains a need for a quantitative evaluation of the array of circulating NO metabolites generated by breathing NO and the fate of these metabolites after ceasing its administration. Furthermore, little is known whether inhaled NO affects the cutaneous microcirculation. We hypothesized that inhaled NO increases the plasma levels of nitrite and SNO-Hb which then modify the cutaneous microvascular response to vasoactive mediators that challenge the NO pathway.

Materials and methods

a) Study approval

The study was approved by the institutional review board of the Cleveland Clinic (IRB # 12–1328). All subjects provided written informed consent prior to inclusion in the study. We conducted this cross-sectional study between May 2015 and September 2015.

b) Subject selection and environment

Volunteers were in excellent general health and underwent a detailed evaluation to rule out smoking or conditions (e.g. diabetes, hypercholesterolemia or hypertension) that could affect the NO metabolism or cutaneous microcirculation. Subjects did not eat or drink for 4 hours prior to testing. Procedures were performed in a private room where individuals were acclimatized for at least 30 minutes (room temperature: 72˚ F or 22˚ C).

c) Nitric oxide inhalation

Nitric oxide (INOMAX, Mallinckrodt Pharmaceuticals, Hazelwood, MO, USA) was administered continuously for 15 minutes through a disposable nasal cannula at a dose of 40 ppm, carried by room air at a flow rate of 4 L/min, following manufacturer's recommendations.

d) Laboratory determinations

-**Blood collection.** We placed a temporary venous access in the dorsal aspect of the right hand. Blood was obtained at baseline (before NO inhalation), at 15 minutes of continuous NO administration and at 5, 15 and 30 minutes of stopping this gas supplementation. Blood was collected using heparin vacutainer tubes (BD Biosciences, NJ, USA) and immediately centrifuged for 3 minutes to separate plasma from red blood cells (RBC). Without any delay, both plasma and RBC samples were frozen using liquid nitrogen, to preserve the NO metabolites. Samples were stored at -80˚ Celsius until assayed. Samples for analysis were only thawed once and the remainder discarded. No detectable nitrite was observed in the heparin vacutainer tubes or in sample storage tubes. All precautions were taken to minimize nitrite loss during the freezing / thawing procedure [41]. In addition, we tested the stability of nitrite levels during the freezing / thawing procedure by measuring nitrite before freezing and after snap freezing and thawing from a single individual and noted only a small variation in the nitrite concentration (fresh sample: 100.1 +2.6 nM and thawed sample: 101.4 +/- 4.8 nM (S1 Table)).

-**Nitrite and nitrate determinations in plasma.** Samples were deproteinated using methanol precipitation. Immediately after thawing the plasma samples, two volumes of methanol were added, and samples kept at -20˚ Celsius for at least 15 minutes to allow protein precipitation. Samples were then spun down to remove the precipitated proteins. The supernatant was then used for subsequent analyses. Nitrite was measured using ozone-based chemiluminescence with the triiodide method and Sievers NO analyzer (GE Analytical Instruments, Boulder, CO, USA) [42–44]. Briefly, the triiodide reagent was made using 1g of KCl and 0.65 g of iodine dissolved in 20 ml of distilled water, with the addition of 70 ml of acetic acid. A total of 6 ml of this solution was used in the reaction chamber for nitrite conversion into NO. A standard curve was generated using up to 500 nM of nitrite. We used 200ul aliquots of the sample to inject into the reaction chamber. Since the volume of the reaction chamber would alter over time, we limited the number of injections but kept the samples from each subject together. At the beginning with each fresh reagent in the reaction chamber, we ran a standard and one after the last sample. The levels of these standards were similar and so suggested the reagent was still sufficiently active. A similar method was used for detection of nitrate, with the only difference that the

reagent was a saturated solution of vanadium (III) chloride (0.6 g in 100 ml of 1N HCl), heated to 94˚ C [45, 46]. A standard curve was generated using nitrate up to 25 uM.

-**SNO-Hb and Hb:[Fe]NO measurement in RBCs.** RBC samples were assayed for total, Fe-bound and thiol-bound NO (SNO) content by photolysis/chemiluminescence, as described previously [47, 48]. Briefly, samples were thawed (2 min, 37C), fully lysed (addition of ddH$_2$O/vortex), and centrifuged (25,000 g, 5 min, 4C) to remove cell debris and membrane. Supernatant was run through a Sephadex G25 spin column, following which [Hb] was measured [49]. To selectively remove thiol bound NO (SNO), samples were incubated ± HgCl$_2$ (6 fold molar excess over [Hb thiol]; tetramer basis)[50, 51]. Following this minimal processing, samples were injected into an HPLC pump (Analytical Scientific Instruments, Richmond, CA) purged with degassed, deionized water as the mobile phase, and carried (1 ml/min) to a custom photolysis system (Technosoft, Morrisville, NC, USA) comprising a quartz coil looped around a mercury arc lamp (Hanovia, Inc., Newark, NJ). This photolytically liberates bound NO, that is then subsequently carried in an inert gas stream (helium), to a high-resolution chemiluminescence NO analyzer (TEA 810, Ellutia, Charleston, SC, USA). Aqueous phase and higher oxides of nitrogen were removed in a series of cold traps interposed between the photolysis and analyzer units. A GSNO standard curve was performed each day with analysis of sample area under curve (Clarity, Madison, CT). Unknown sample areas were read off the standard curve, to determine NO content. Total NO content was derived from vehicle incubated sample. Fe-NO was obtained from the sample incubated with HgCl$_2$. SNO-Hb was calculated from the difference between these two signals. Measurements of RBC NO are presented as a molar ratio of NO to Hb. Determinations were done in duplicate and an average reported [47, 48].

-**Bicarbonate determination.** Bicarbonate was measured using a Bicarbonate reagent kit on a Cobas C501 analyzer (Roche Diagnostics, Indianapolis, IN). Briefly, phosphoenolpyruvate in the presence of phosphoenolpyruvate carboxylase to produces oxaloacetate and phosphate. The oxaloacetate produced is coupled with NADH in the presence of malate dehydrogenase to produce malate and NAD. The consumption of NADH is measured 320 nm to 400 nm and the bicarbonate concentration determined.

-**Cutaneous microvascular studies.** Subjects were tested in a sitting position with the forearm at the level of the heart. We measured the subjects' heart rate, blood pressure, and pulse oxygen saturation. We carefully prepped the skin of the anterior aspect of the forearm, 5 cm distal to the antecubital fossa and away from visible veins or skin abrasion [52]. Great care was taken to create similar experimental conditions to ensure regional and temporal reproducibility.

a) Skin microvascular flow

We estimated the skin microvascular flow during the last minute of NO inhalation and for 5 minutes after its discontinuation, using Laser Doppler flowmetry [53] with the PeriFlux system 5000 (Perimed, Järfälla, Sweden) and an integrating probe (PF 413) [54]. We continuously recorded data from the instrument for off-line review, using the PeriSoft for Windows software. Flow measurements are expressed as arbitrary perfusion units (PU) averaged over 30 seconds of recording. Probes were calibrated using a motility standard consisting of a colloidal suspension of polystyrene particles (PF 1000). Laser Doppler flowmetry assesses the skin capillary perfusion (depth of skin penetration of about 1mm) by measuring the Doppler shift induced by the laser light scattering of moving red blood cells. Perfusion is defined as the concentration of red blood cells times their average velocity [55, 56].

b) Iontophoresis of acetylcholine and sodium nitroprusside

Iontophoresis was performed using the thermostatic probe PF 481, the periIont micropharmacology system, the drug delivery electrodes PF 383 and the dispersive electrodes PF 384

(Perimed, Järfälla, Sweden) [52]. At different skin sites, we placed 180 μL of either acetylcholine 1% (Sigma-Aldrich, St Louis, MO, USA) or sodium nitroprusside 1% (Marathon Pharmaceuticals, LLC, Northbrook, IL, USA) in the sponge of the drug-delivery electrode. We iontophoresed these agents at 40 uA for 5 minutes. We used a positive polarity for acetylcholine and negative polarity for sodium nitroprusside. Both tests were performed before and during NO administration (at minutes 5 and 9 for acetylcholine and sodium nitroprusside, respectively). Current-induced vasodilation was prevented by limiting the current density to < 0.01 mA/cm^2 [57]. During each test, we measured changes in PU and monitored skin resistance and temperature. Skin resistance was determined by the periIont micropharmacology system and recorded before and during the iontophoresis of medications, since variations in the skin resistance may affect the current flow and the transport of vasoactive mediators.

We believe that the cutaneous iontophoresis of vasoactive agents has not affected the levels of NO metabolites, given its local effect, low dose and the fact that blood was obtained in the opposite arm. Additionally, we have previously shown that using our methodology levels of iontophoresed agents are not detectable in blood [52].

Statistics

Continuous data are presented as median (interquartile range (IQR)). Categorical data are summarized as discrete values and percentages (n (%)). Results of the iontophoresis tests are expressed as peak PUs or percentage of variation from baseline. Independent continuous samples were compared using Wilcoxon-Mann-Whitney test. Paired continuous samples were tested with Wilcoxon signed-rank test and Bonferroni correction. We tested repeated measurements with the nonparametric Friedman test. Relationships between normally distributed variables were assessed using the Spearman rank correlation coefficient. All *p* values are two-tailed and a value of < 0.05 was considered significant. The statistical analyses were performed using the statistical package IBM SPSS, version 20 (IBM; Armonk, New York) and MedCalc, version 14.12.0 (Ostend, Belgium).

Results

a) Nitric oxide metabolites

We included 15 subjects with a median (IQR) age of 37 (31–47) years, of whom 13 (87%) were females. We tested nitrite, nitrate, SNO-Hb and Hb:[Fe]NO before, during and after NO administration (Table 1 and Fig 1). The basal levels of nitrite and SNO-Hb are consistent to those found by previous investigators [48, 58–61]. The Friedman test showed significant differences among the various time points in nitrite, total RBC NO, Hb:[Fe]NO and SNO-Hb (Table 1). We observed a significant increase in nitrite (median (IQR) difference of 7 (-2 –+19) %, p = 0.048), total RBC NO (29 (-12 –+111) %, p = 0.02) and SNO-Hb (median (IQR) difference of 59 (-15 - +282) %, p = 0.02) during NO inhalation compared to baseline. Nitrite, total RBC NO and SNO-Hb levels were higher 5 minutes after the discontinuation of NO compared to baseline (28 (13–52) %, p = 0.009, 46 (3–96) %, p = 0.006, and 50 (-16 –+329) %, p = 0.03, respectively). Interestingly, the levels of nitrite peaked at 5 minutes; meanwhile, total RBC NO and SNO-Hb continued to increase during the 30 minutes after discontinuing NO.

b) Bicarbonate determinations

In 11 subjects we measured bicarbonate in plasma before and 30 minutes after NO inhalation. The median (IQR) before NO inhalation and 30 minutes after discontinuation were 20.6 (19.8–23.4) and 20.5 (18.5–20.8), respectively (p = 0.04). The change in bicarbonate at 30

Table 1. Determinations of NO metabolites in plasma and RBC.

		Baseline Median (IQR)	During NO inhalation* Median (IQR)	After 5 min of NO discontinuation Median (IQR)	After 15 min of NO discontinuation Median (IQR)	After 30 min of NO discontinuation Median (IQR)	P (Friedman test)
Plasma	n	15	15	15	15	14^	
	Nitrate (uM)	25.4 (20.9–35.0)	26.6 (21.4–35.4)	27.3 (22.8–35.3)	27.4 (20.8–33.7)	26.9 (20.2–34.3)	0.07
	Nitrite (nM)	102.8 (86.3–117.8)	114.0 (87.0–129.0)	131.3 (104.3–169.5)	119.3 (84.8–141.8)	115.5 (105.8–127.5)	<0.001
RBC	**Total NO** (moles NO:Hb x 10^{-3})	13.2 (8.0–15.7)	15.3 (11.4–26.2)	15.2 (9.9–21.6)	18.2 (11.3–32.6)	18.7 (12.8–33.1)	0.005
	NO[Fe]Hb (moles NO:Hb x 10^{-3})	7.1 (3.0–11.8)	7.1 (3.7–14.6)	9.0 (2.8–12.2)	8.2 (4.7–9.8)	8.3 (5.4–19.0)	<0.001
	SNO-Hb (moles NO:Hb x 10^{-3})	4.7 (2.5–5.8)	7.8 (4.1–13.1)	7.3 (4.5–14.8)	10.3 (5.6–18.2)	10.6 (5.3–15.5)	0.005

*blood sample obtained at 15 minutes of continuous inhaled NO administration.

^ One patient had no blood sample 30 minutes after NO discontinuation.

Abbreviations: Hb:[Fe]NO: nitrosyl Hb, IQR interquartile range, NO: nitric oxide, O2: oxygen, SNO-Hb: S-nitrosothiol Hb.

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minutes was negatively associated with the change in SNO-Hb; however this association did not reach statistical significance.

c) Forearm microvascular studies

The skin microvascular flow was not significantly different at baseline than during NO inhalation (Table 2). During NO inhalation, compared to before NO inhalation, the peak PU increased 62.8 (-218 - + 333.7) % points during the iontophoresis of acetylcholine and 95.8 (-123 - + 262.1) % points during the iontophoresis of sodium nitroprusside; changes that did not achieve statistical significance (Table 2).

Before NO inhalation, the peak PU and increase in PU during acetylcholine iontophoresis showed a negative association with the level of SNO-Hb (r: -0.57, p = 0.03 and r: -0.61, p = 0.03, respectively). Similarly, during NO administration, the peak PU and increase in PU during acetylcholine iontophoresis was negatively associated to the level of SNO-Hb under the same condition (r: -0.54, p = 0.04 and r: -0.51, p = 0.05, respectively). The change in peak PU during acetylcholine before and during NO inhalation was inversely related to the change in SNO-Hb (r: -0.57, p = 0.03). The levels of nitrite were not significantly associated with the acetylcholine iontophoresis response before or during NO administration.

Discussion

It is postulated that many of the systemic responses to inhaled NO are derived from its metabolites such as nitrite, S-nitrosothiols and possibly dinitrosyl iron complexes (DNICs). Both nitrite and S-nitrosothiols can be converted back to NO to deliver this molecule to sites remote to its generation. Nitrite can be reduced to NO by nitrite reductases and by low pH in ischemic tissues, whereas SNO-Hb can transfer NO^+ under low oxygen concentrations in the peripheral circulation [14] (Fig 2). In the present study, we investigated how these metabolites changed during and 30 minutes after discontinuing a 15 minute inhalation of NO. To the best of our knowledge, the kinetics of inhaled NO increasing levels of SNO-Hb, have not been measured. We noted that the inhalation of NO led to a significant increase in nitrite and SNO-Hb. Nitrite peaked at 5 minutes and SNO-Hb peaked beyond 15 minutes, of discontinuing the NO
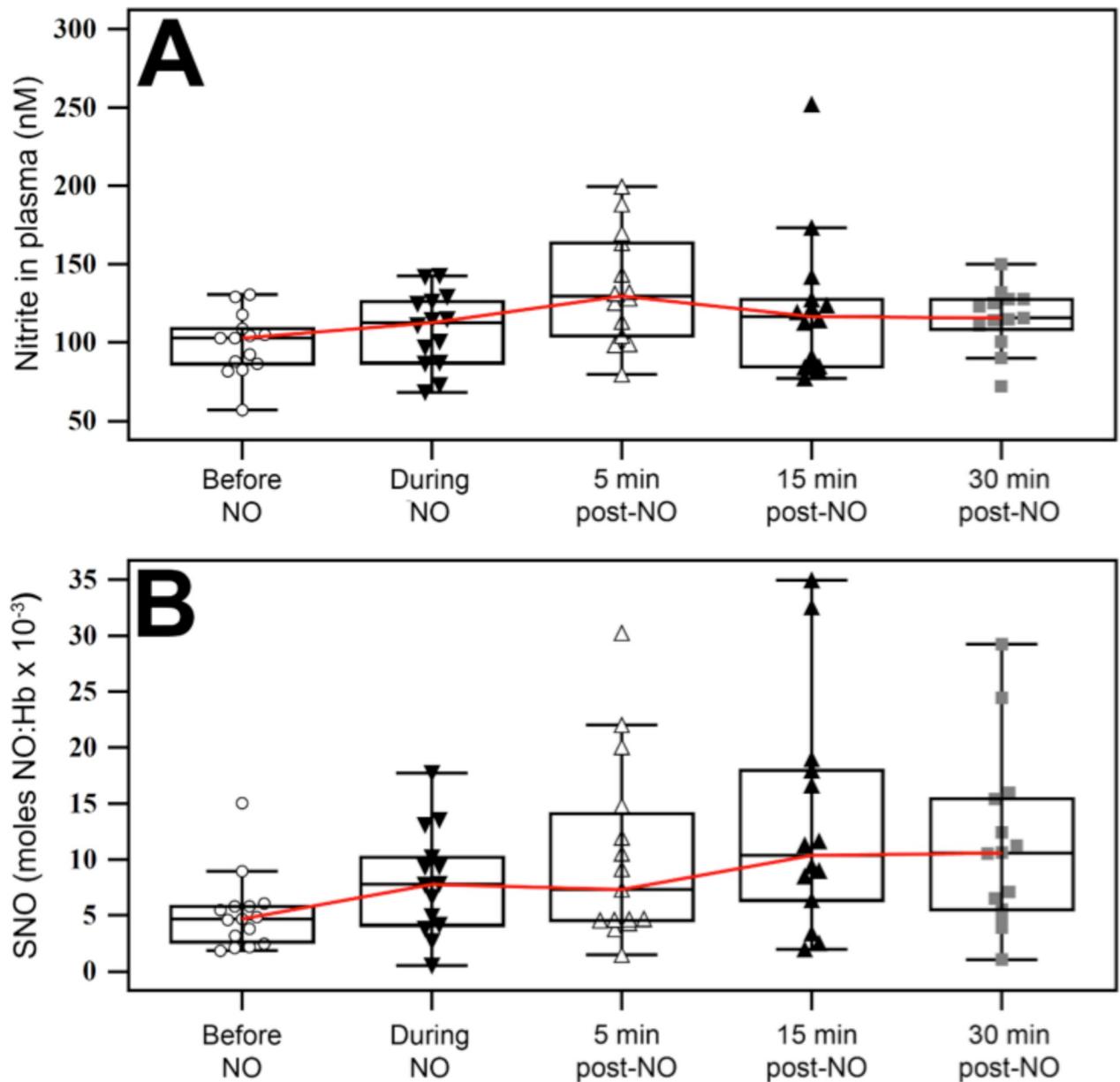
Fig 1. Box plots of plasma nitrite and RBC SNO-Hb before, during and after NO inhalation. The red line connects the median values at the different time points.

inhalation. In fact, nitrite levels rapidly declined after its peak (consistent with the suggested half-life of 30 minutes) [59, 62]; while SNO-Hb levels remained elevated for at least 30 minutes after NO discontinuation, theoretically storing the NO signal for a longer time and possibly increasing the minute ventilation.

Conventionally, most excess inhaled NO reacts with Fe^{++} Hb and oxygen to form methemoglobin and nitrate; the methemoglobin is then recycled by methemoglobin reductases [40, 63]. Strikingly, we did not notice a significant change in plasma nitrate during NO inhalation. Many factors can affect nitrate levels, including diet and microbiome [64]. Inhalation of NO at the concentration given would produce at best a small and likely non-significant change given the inherent noise of the assay used.

Table 2. Forearm microvascular studies at baseline and during NO inhalation.

Variables	Baseline Median (IQR)	During NO inhalation Median (IQR)	P (Wilcoxon signed-rank test)
Cutaneous PU	13.6 (11.8–16.8)	14.6 (9.8–20.6)	0.28
Acetylcholine iontophoresis			
Baseline PU	5.6 (4.3–7.2)	4.5 (3.6–5.5)	0.33
Peak PU	49.6 (28.2–60.8)	47.4 (37.6–51.8)	0.87
Percentage change in PU	600 (420–965)	991 (363–1249)	0.57
Skin resistance	215 (175–279)	207 (165–261)	0.42
Sodium nitroprusside iontophoresis			
Baseline PU	4.1 (3.6–7.6)	4.2 (2.8–5.3)	0.31
Peak PU	17.1 (10.3–21.1)	11.6 (7.1–25.9)	0.96
Percentage change in PU	190 (127–407)	231 (107–424)	0.36
Skin resistance	241 (220–251)	222 (208–272)	0.49

In mice, the inhalation of NO led to increases in nitrate and nitrite in plasma as well as SNO-Hb and Hb:[Fe]NO in erythrocytes [65], with a plateau achieved within 15 minutes of administering NO [65]. In patients undergoing liver transplantation [66], inhaled NO produced an increase in plasma nitrate and nitrite as well as erythrocyte Hb:[Fe]NO, but not
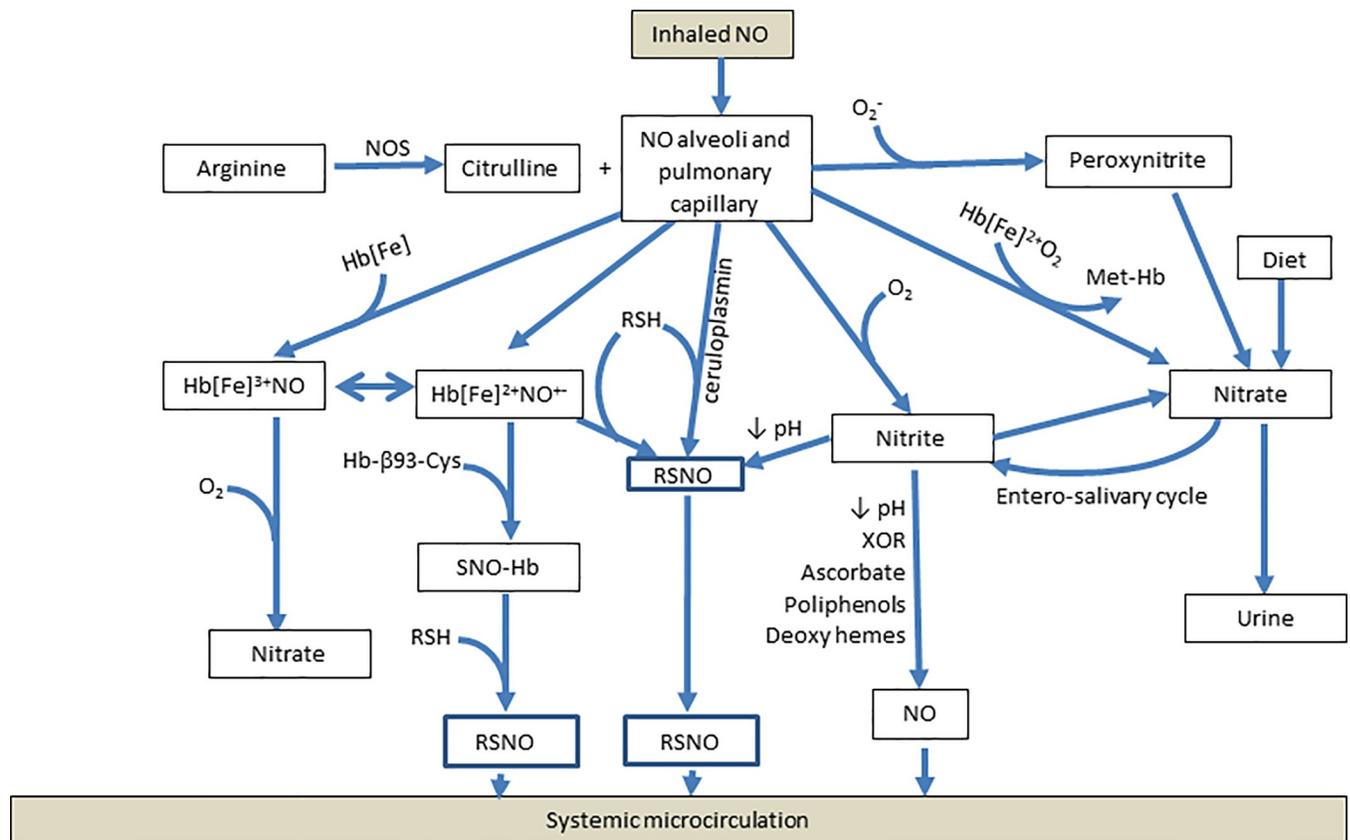

Fig 2. Mechanisms by which inhaled NO acts on the systemic microcirculation. Abbreviations: Hb: hemoglobin, Hb:[Fe]NO: nitrosyl Hb, Met-Hb: methemoglobin, NO: nitric oxide, NOS: nitric oxide synthase, O_2: oxygen, ROS: reactive oxygen species, RSH: alkyl thiols, RSNO: S nitrosothiols, SNO-Hb: S-nitrosothiol Hb, XOR: xanthine oxidoreductase.

SNO-Hb. In contrast, our study showed an increase in SNO-Hb during NO inhalation, and the discrepancy may be in part related to the difficulties in measuring SNO-Hb using iodine-based methods [67], NO dosing and length of NO administration.

Initial studies suggested that the effects of inhaled NO were confined to the lungs given its high affinity for the heme moiety of Hb [68]. Subsequent investigations, in animals, showed that inhaled NO inhibits platelet function [69], increases glomerular filtration rate[70], reduces neointimal formation in injured arteries [71], enhances coronary artery patency after thrombolysis [72], reduces myocardial infarction size [73], and maintains mesenteric blood flow in intestinal ischemia-reperfusion injury [74]. Meanwhile, in humans, inhaled NO was noted to reduce muscle inflammation during limb ischemia [75] and ischemia-reperfusion injury during liver transplantation [66]. Of concern, long-term follow-up of infants exposed to inhaled NO suggests an increased cancer risk later in childhood [27]. Nitric oxide itself can be mutagenic, but most authors argue that NO radical is not present in any relevant concentration in the presence of mM vascular concentrations of Hb. The exception may be in ischemic tissue, where nitrite could be converted to NO by protonation. Thus, the peripheral oncogenic effects of inhaled NO could result from delivery of nitrite to ischemic tissues [76]. The peripheral physiologic effects of inhaled NO are typically dose- and oxygen tension-dependent and can occur without systemic hemodynamic changes [26, 77].

Nitric oxide equivalents are transferred as NO^+ from deoxyhemoglobin to low molecular weight thiols in erythrocytes [14, 78–81]. These low molecular weight S-nitrosothiols are hypoxia-mimetic, signaling Hb R to T conformational change [14, 78–81]. One of these hypoxia-mimetic effects is to increase minute ventilation [78, 82, 83]. To maintain pH, hyperventilation-induced respiratory alkalosis will normally result in decreased bicarbonate levels. A decrease in steady state bicarbonate of 0.1 to maintain steady-state pH, by Henderson-Hasselbalch, corresponds to a decrease in steady state pCO_2 of 0.5 mmHg. By Dubois, this corresponds to a steady increase in minute ventilation of ~ 0.5 litres per minute at steady state pO_2. Though this normal physiology is not observed in critically ill patients who are mechanically ventilated, we were able here, uniquely, to measure the decrease in bicarbonate associated with excess NO-hemoglobin loading in spontaneously breathing, healthy subjects.

The iontophoresis of acetylcholine and sodium nitroprusside test the microvascular endothelial-dependent and independent vasodilation, respectively [84]. Acetylcholine induces vasodilation via the endothelial production of NO and prostanoids, pathways that likely participate in enzymatic cross-talk [85]. Sodium nitroprusside reacts with tissue sulfhydryl groups to produce NO directly (NO donor) [85]. In our study, the inhalation of NO did not significantly affect the microvascular cutaneous perfusion or the response to the iontophoresis of acetylcholine or sodium nitroprusside. These findings could represent that the microvascular studies used were not sensitive and/or precise enough to detect the systemic microvascular effects of inhaled NO, and/or the normal tissue oxygen tension of skin tissue. Another explanation could be that inhaled NO elicits a more pronounced microvascular response in states of oxidative stress, hypoxic tissues or decreased NO production than in healthy controls [74]. In fact, Cannon III et al [40] noted that NO inhalation produced minimal effects on the forearm blood flow; however, inhaled NO reverted the reduced forearm blood flow caused by the blockade of NO synthesis with NG-monomethyl-L-arginine [40].

Prior investigations in healthy individuals showed that stimulation with acetylcholine augmented forearm blood flow and venous nitrite [64, 86]. Although we showed no significant changes in the microvascular studies during the inhalation of NO, we noted an inverse association between the levels of SNO-Hb and the response to acetylcholine iontophoresis both before and during NO administration. A higher RBC level of SNO-Hb may represent a higher NO state with less microvascular response to the iontophoresis of acetylcholine. Interestingly, the

more pronounced the increase in SNO-Hb during NO inhalation the lower the change in response to acetylcholine iontophoresis between baseline and NO administration.

Note that under conditions of extreme oxidative stress in the lungs, i.e. acute respiratory distress syndrome, inhaled NO has adverse effects on renal function, whereas during heart surgery without lung injury, inhaled NO may benefit renal function [28, 29]. This difference suggests that airway chemistry is important to the metabolic fate and systemic effects of inhaled NO [14] (Fig 2). Consistent with this hypothesis, SNO-Hb levels continued to increase after the inhaled NO was discontinued. The duration of this increase was longer than a circulatory cycle, arguing against simple transfer of NO from Fe^{++} Hb to Hb-thiol as the principle reason for this steady post-dose increase [26]; and stoichiometrically, the increase in SNO-Hb occurred without any loss of RBC NO. These data almost certainly argue for a capacitor in the circuit: a reservoir of SNO in the airways that stores NO^+ equivalents and transfers them to the blood. In fact, airway reduced thiol levels are in excess of 100 μM, and formation of a major SNO reservoir from endogenous thiols has previously been demonstrated in the distal airway during NO inhalation in humans and in pigs [87, 88].

Limitations of the current study include a) a relatively small number of healthy volunteers (n = 15) that might have prevented the identification of significant differences in microvascular studies or NO metabolites, and b) microvascular studies were not repeated after the discontinuation of NO when the levels of nitrite and SNO-Hb peaked. However, for the first time, we rigorously measured in healthy volunteers both nitrite and SNO-Hb at baseline, during and up to 30 minutes after the discontinuation of NO inhalation. Our data show that both nitrite in plasma and SNO-Hb in RBC increase during and immediately after NO inhalation and that these metabolites may affect studies that test the NO pathway in the systemic microvasculature.

Conclusions

For the first time we measured the kinetics of nitrite and SNO-Hb during NO inhalation and after its discontinuation. Interestingly, the kinetics of these NO metabolites are not identical. Nitrite increases first, followed by a more sustained increase in Hb-SNO, likely reflecting the capacitor-like reservoir of SNO in the lung. Nitrite, Hb-SNO and possibly DNICs could be a mobile reservoir of NO with implications on the systemic microvasculature.

Supporting information

S1 Table. Effect of freezing/thawing on nitrite measurements. This is a table with the measurements of nitrite from samples that were fresh or frozen/thawed from a single individual. This demonstrated that the nitrite levels were not affected by freezing and thawing under the condition we used.
(XLSX)

S2 Table. Measurement of SNO, Nitrite, and Nitrate. This excel sheet contains the data for the measurements of SNO, nitrite and nitrate for the different samples. It also contains the standard curves along with the associated R^2 values and the calculated values of the samples.
(XLSX)

S3 Table. Collated data for NOx metabolites, CO_2 and iontophoresis related measurements. In addition to the different NOx metabolites, CO_2 and iontophoresis results it also includes data related to age, gender and BMI status of the subjects.
(XLSX)

Author Contributions

Conceptualization: Adriano R. Tonelli, Raed A. Dweik.

Data curation: Adriano R. Tonelli, Kulwant S. Aulak, Mostafa K. Ahmed, Alfred Hausladen, Batool Abuhalimeh, Charlie J. Casa, Stephen C. Rogers, David Timm, Allan Doctor, Benjamin Gaston.

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References

1. Culotta E, Koshland DE Jr., NO news is good news. Science. 1992; 258(5090):1862–5.

2. Daiber A, Xia N, Steven S, Oelze M, Hanf A, Kroller-Schon S, et al. New Therapeutic Implications of Endothelial Nitric Oxide Synthase (eNOS) Function/Dysfunction in Cardiovascular Disease. International journal of molecular sciences. 2019;20(1).

3. Rosselli M, Keller PJ, Dubey RK. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Human reproduction update. 1998; 4(1):3–24. https://doi.org/10.1093/humupd/4.1.3 PMID: 9622410

4. Stuehr DJ, Marletta MA. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proceedings of the National Academy of Sciences of the United States of America. 1985; 82(22):7738–42. https://doi.org/10.1073/pnas.82.22.7738 PMID: 3906650

5. Tonelli AR, Haserodt S, Aytekin M, Dweik RA. Nitric oxide deficiency in pulmonary hypertension: Pathobiology and implications for therapy. Pulm Circ. 2013; 3(1):20–30. https://doi.org/10.4103/2045-8932.109911 PMID: 23662172

6. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980; 288(5789):373–6. https://doi.org/10.1038/288373a0 PMID: 6253831

7. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proceedings of the National Academy of Sciences of the United States of America. 1987; 84(24):9265–9. https://doi.org/10.1073/pnas.84.24.9265 PMID: 2827174

8. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothe-lium-derived relaxing factor. Nature. 1987; 327(6122):524–6. https://doi.org/10.1038/327524a0 PMID: 3495737

9. Coggins MP, Bloch KD. Nitric oxide in the pulmonary vasculature. Arterioscler Thromb Vasc Biol. 2007; 27(9):1877–85. https://doi.org/10.1161/ATVBAHA.107.142943 PMID: 17541026

10. Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J. 1992; 6(12):3051–64. PMID: 1381691

11. Liu X, Miller MJ, Joshi MS, Sadowska-Krowicka H, Clark DA, Lancaster JR Jr., Diffusion-limited reaction of free nitric oxide with erythrocytes. The Journal of biological chemistry. 1998; 273(30):18709–13. https://doi.org/10.1074/jbc.273.30.18709 PMID: 9668042

12. Kelm M, Schrader J. Control of coronary vascular tone by nitric oxide. Circ Res. 1990; 66(6):1561–75. https://doi.org/10.1161/01.res.66.6.1561 PMID: 2160870

13. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993; 329(27):2002–12. PMID: 7504210

14. Marozkina NV, Gaston B. Nitrogen chemistry and lung physiology. Annual review of physiology. 2015; 77:431–52. https://doi.org/10.1146/annurev-physiol-021113-170352 PMID: 25668023

15. Kinsella JP, Neish SR, Shaffer E, Abman SH. Low-dose inhalation nitric oxide in persistent pulmonary hypertension of the newborn. Lancet. 1992; 340(8823):819–20. PMID: 1357246

16. Roberts JD, Polaner DM, Lang P, Zapol WM. Inhaled nitric oxide in persistent pulmonary hypertension of the newborn. Lancet. 1992; 340(8823):818–9. https://doi.org/10.1016/0140-6736(92)92686-a PMID: 1357245

17. Kinsella JP, Abman SH. Inhaled nitric oxide in the premature infant: animal models and clinical experi-ence. Seminars in perinatology. 1997; 21(5):418–25. PMID: 9352614

18. Roberts JD Jr., Fineman JR, Morin FC 3rd, Shaul PW, Rimar S, Schreiber, et al. Inhaled nitric oxide and persistent pulmonary hypertension of the newborn. The Inhaled Nitric Oxide Study Group. The New England journal of medicine. 1997; 336(9):605–10. https://doi.org/10.1056/NEJM199702273360902 PMID: 9032045

19. Bhat T, Neuman A, Tantary M, Bhat H, Glass D, Mannino W, et al. Inhaled nitric oxide in acute pulmo-nary embolism: a systematic review. Reviews in cardiovascular medicine. 2015; 16(1):1–8. PMID: 25813791

20. Gonzalez A, Fabres J, D'Apremont I, Urcelay G, Avaca M, Gandolfi C, et al. Randomized controlled trial of early compared with delayed use of inhaled nitric oxide in newborns with a moderate respiratory fail-ure and pulmonary hypertension. Journal of perinatology: official journal of the California Perinatal Association. 2010; 30(6):420–4.

21. Hajian B, De Backer J, Vos W, Van Holsbeke C, Ferreira F, Quinn DA, et al. Pulmonary vascular effects of pulsed inhaled nitric oxide in COPD patients with pulmonary hypertension. International journal of chronic obstructive pulmonary disease. 2016; 11:1533–41. https://doi.org/10.2147/COPD.S106480 PMID: 27462149

22. Stewart DL, Vogel PA, Jarrett B, Potenziano J. Effect of inhaled nitric oxide on oxygen therapy, mechan-ical ventilation, and hypoxic respiratory failure. Minerva pediatrica. 2018; 70(1):51–8. https://doi.org/10.23736/S0026-4946.17.04944-1 PMID: 28472876

23. Yu B, Ichinose F, Bloch DB, Zapol WM. Inhaled nitric oxide. British journal of pharmacology. 2019; 176 (2):246–55. https://doi.org/10.1111/bph.14512 PMID: 30288739

24. Tonelli AR, Alnuaimat H, Mubarak K. Pulmonary vasodilator testing and use of calcium channel block-ers in pulmonary arterial hypertension. Respiratory medicine. 2010; 104(4):481–96. https://doi.org/10.1016/j.rmed.2009.11.015 PMID: 20004088

25. Wagner HM. Absorption von NO und NO2 in MIK-und MAK-Konzentrationen bei der Inhalation. Luft. 1970; 30:380–1.

26. McMahon TJ, Doctor A. Extrapulmonary effects of inhaled nitric oxide: role of reversible S-nitrosylation of erythrocytic hemoglobin. Proceedings of the American Thoracic Society. 2006; 3(2):153–60. https://doi.org/10.1513/pats.200507-066BG PMID: 16565424

27. Dixon F, Ziegler DS, Bajuk B, Wright I, Hilder L, Abdel Latif ME, et al. Treatment with nitric oxide in the neonatal intensive care unit is associated with increased risk of childhood cancer. Acta paediatrica. 2018; 107(12):2092–8. https://doi.org/10.1111/apa.14436 PMID: 29873414

28. Ruan SY, Wu HY, Lin HH, Wu HD, Yu CJ, Lai MS. Inhaled nitric oxide and the risk of renal dysfunction in patients with acute respiratory distress syndrome: a propensity-matched cohort study. Critical care. 2016; 20(1):389. https://doi.org/10.1186/s13054-016-1566-0 PMID: 27903300

29. Lei C, Berra L, Rezoagli E, Yu B, Dong H, Yu S, et al. Nitric Oxide Decreases Acute Kidney Injury and Stage 3 Chronic Kidney Disease after Cardiac Surgery. Am J Respir Crit Care Med. 2018; 198 (10):1279–87. https://doi.org/10.1164/rccm.201710-2150OC PMID: 29932345

30. Brucken A, Derwall M, Bleilevens C, Stoppe C, Gotzenich A, Gaisa NT, et al. Brief inhalation of nitric oxide increases resuscitation success and improves 7-day-survival after cardiac arrest in rats: a randomized controlled animal study. Critical care. 2015; 19:408. https://doi.org/10.1186/s13054-015-1128-x PMID: 26577797

31. Derwall M, Ebeling A, Nolte KW, Weis J, Rossaint R, Ichinose F, et al. Inhaled nitric oxide improves transpulmonary blood flow and clinical outcomes after prolonged cardiac arrest: a large animal study. Critical care. 2015; 19:328. https://doi.org/10.1186/s13054-015-1050-2 PMID: 26369409

32. Kida K, Ichinose F. Preventing ischemic brain injury after sudden cardiac arrest using NO inhalation. Critical care. 2014; 18(2):212. https://doi.org/10.1186/cc13779 PMID: 25029464

33. Terpolilli NA, Feiler S, Dienel A, Muller F, Heumos N, Friedrich B, et al. Nitric oxide inhalation reduces brain damage, prevents mortality, and improves neurological outcome after subarachnoid hemorrhage by resolving early pial microvasospasms. Journal of cerebral blood flow and metabolism: official journal of the International Society of Cerebral Blood Flow and Metabolism. 2016; 36(12):2096–107.

34. Terpolilli NA, Kim SW, Thal SC, Kataoka H, Zeisig V, Nitzsche B, et al. Inhalation of nitric oxide prevents ischemic brain damage in experimental stroke by selective dilatation of collateral arterioles. Circulation research. 2012; 110(5):727–38. https://doi.org/10.1161/CIRCRESAHA.111.253419 PMID: 22207711

35. Terpolilli NA, Kim SW, Thal SC, Kuebler WM, Plesnila N. Inhaled nitric oxide reduces secondary brain damage after traumatic brain injury in mice. Journal of cerebral blood flow and metabolism: official journal of the International Society of Cerebral Blood Flow and Metabolism. 2013; 33(2):311–8.

36. Singel DJ, Stamler JS. Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin. Annu Rev Physiol. 2005; 67:99–145. PMID: 15709954

37. Lundberg JO, Weitzberg E. Nitrite reduction to nitric oxide in the vasculature. Am J Physiol Heart Circ Physiol. 2008; 295(2):H477–8. https://doi.org/10.1152/ajpheart.00611.2008 PMID: 18586886

38. Alzawahra WF, Talukder MA, Liu X, Samouilov A, Zweier JL. Heme proteins mediate the conversion of nitrite to nitric oxide in the vascular wall. Am J Physiol Heart Circ Physiol. 2008; 295(2):H499–508. https://doi.org/10.1152/ajpheart.00374.2008 PMID: 18539756

39. Gaston B. Summary: systemic effects of inhaled nitric oxide. Proc Am Thorac Soc. 2006; 3(2):170–2. https://doi.org/10.1513/pats.200506-049BG PMID: 16565427

40. Cannon RO 3rd, Schechter AN, Panza JA, Ognibene FP, Pease-Fye ME, Waclawiw MA, et al. Effects of inhaled nitric oxide on regional blood flow are consistent with intravascular nitric oxide delivery. The Journal of clinical investigation. 2001; 108(2):279–87. https://doi.org/10.1172/JCI12761 PMID: 11457881

41. Daiber A, Bachschmid M, Kavakli C, Frein D, Wendt M, Ullrich V, et al. A new pitfall in detecting biological end products of nitric oxide-nitration, nitros(yl)ation and nitrite/nitrate artefacts during freezing. Nitric oxide: biology and chemistry / official journal of the Nitric Oxide Society. 2003; 9(1):44–52.

42. Hausladen A, Rafikov R, Angelo M, Singel DJ, Nudler E, Stamler JS. Assessment of nitric oxide signals by triiodide chemiluminescence. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104(7):2157–62. https://doi.org/10.1073/pnas.0611191104 PMID: 17287342

43. Pinder AG, Rogers SC, Khalatbari A, Ingram TE, James PE. The Measurement of Nitric Oxide and Its Metabolites in Biological Samples by Ozone-Based Chemiluminescence. In: Hancock JT, editor. Redox-Mediated Signal Transduction: Methods and Protocols. Totowa, NJ: Humana Press; 2009. p. 10–27.

44. Samouilov A, Zweier JL. Development of chemiluminescence-based methods for specific quantitation of nitrosylated thiols. Analytical biochemistry. 1998; 258(2):322–30. https://doi.org/10.1006/abio.1998.2609 PMID: 9570848

45. Braman RS, Hendrix SA. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium (III) reduction with chemiluminescence detection. Analytical chemistry. 1989; 61 (24):2715–8. https://doi.org/10.1021/ac00199a007 PMID: 2619057

46. Cox RD. Determination of nitrate and nitrite at the parts per billion level by chemiluminescence. Anal Chem 1980; 52(2):332–5.

47. Saville B. A scheme for the colorimetric determination of microgram amounts of thiols. Analyst. 1958; 83:670–72.

48. Gow A, Doctor A, Mannick J, Gaston B. S-Nitrosothiol measurements in biological systems. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2007; 851(1–2):140–51. https://doi.org/10.1016/j.jchromb.2007.01.052 PMID: 17379583

49. Drabkin DL, Austin JH. Spectro-photometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. J Biol Chem. 1935; 112:51–65.

50. McMahon TJ, Moon RE, Luschinger BP, Carraway MS, Stone AE, Stolp BW, et al. Nitric oxide in the human respiratory cycle. Nature medicine. 2002; 8(7):711–7. https://doi.org/10.1038/nm718 PMID: 12042776

51. Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. Nature. 1996; 380(6571):221–6. https://doi.org/10.1038/380221a0 PMID: 8637569

52. Tonelli AR, Ahmed MK, Alkukhun L, Cikach F, Aulak K, Dweik RA. Treprostinil Iontophoresis in Idiopathic Pulmonary Arterial Hypertension. American journal of respiratory and critical care medicine. 2015; 192(8):1014–6. https://doi.org/10.1164/rccm.201506-1091LE PMID: 26469842

53. Stern MD. In vivo evaluation of microcirculation by coherent light scattering. Nature. 1975; 254 (5495):56–8. PMID: 1113878

54. Ahn H, Johansson K, Lundgren O, Nilsson GE. In vivo evaluation of signal processors for laser Doppler tissue flowmeters. Med Biol Eng Comput. 1987; 25(2):207–11. PMID: 2961959

55. Fredriksson I, Larsson M, Stromberg T. Measurement depth and volume in laser Doppler flowmetry. Microvascular research. 2009; 78(1):4–13. https://doi.org/10.1016/j.mvr.2009.02.008 PMID: 19285089

56. Saygin D, Highland KB, Tonelli AR. Microvascular involvement in systemic sclerosis and systemic lupus erythematosus. Microcirculation. 2019; 26(3):e12440. https://doi.org/10.1111/micc.12440 PMID: 29323451

57. Droog EJ, Henricson J, Nilsson GE, Sjoberg F. A protocol for iontophoresis of acetylcholine and sodium nitroprusside that minimises nonspecific vasodilatory effects. Microvasc Res. 2004; 67(2):197–202. https://doi.org/10.1016/j.mvr.2003.12.003 PMID: 15020211

58. Doctor A, Gaston B, Kim-Shapiro DB. Detecting physiologic fluctuations in the S-nitrosohemoglobin micropopulation: Triiodide versus 3C. Blood. 2006; 108(9):3225–6; author reply 6–7. https://doi.org/10.1182/blood-2006-05-026047 PMID: 17057023

59. Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, et al. Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. Circulation. 2007; 116(16):1821–31. https://doi.org/10.1161/CIRCULATIONAHA.107.712133 PMID: 17893272

60. Hon YY, Sun H, Dejam A, Gladwin MT. Characterization of erythrocytic uptake and release and disposition pathways of nitrite, nitrate, methemoglobin, and iron-nitrosyl hemoglobin in the human circulation. Drug metabolism and disposition: the biological fate of chemicals. 2010; 38(10):1707–13.

61. Ibrahim YI, Ninnis JR, Hopper AO, Deming DD, Zhang AX, Herring JL, et al. Inhaled nitric oxide therapy increases blood nitrite, nitrate, and s-nitrosohemoglobin concentrations in infants with pulmonary hypertension. The Journal of pediatrics. 2012; 160(2):245–51. https://doi.org/10.1016/j.jpeds.2011.07.040 PMID: 21907348

62. Rix PJ, Vick A, Attkins NJ, Barker GE, Bott AW, Alcorn H Jr., et al. Pharmacokinetics, pharmacodynamics, safety, and tolerability of nebulized sodium nitrite (AIR001) following repeat-dose inhalation in healthy subjects. Clinical pharmacokinetics. 2015; 54(3):261–72. https://doi.org/10.1007/s40262-014-0201-y PMID: 25421879

63. Westfelt UN, Benthin G, Lundin S, Stenqvist O, Wennmalm A. Conversion of inhaled nitric oxide to nitrate in man. Br J Pharmacol. 1995; 114(8):1621–4. https://doi.org/10.1111/j.1476-5381.1995.tb14948.x PMID: 7599931

64. Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, et al. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. Proc Natl Acad Sci U S A. 2001; 98(22):12814–9. https://doi.org/10.1073/pnas.221381098 PMID: 11606734

65. Nagasaka Y, Fernandez BO, Garcia-Saura MF, Petersen B, Ichinose F, Bloch KD, et al. Brief periods of nitric oxide inhalation protect against myocardial ischemia-reperfusion injury. Anesthesiology. 2008; 109(4):675–82. https://doi.org/10.1097/ALN.0b013e318186316e PMID: 18813047

66. Lang JD Jr., Teng X, Chumley P, Crawford JH, Isbell TS, Chacko BK, et al. Inhaled NO accelerates restoration of liver function in adults following orthotopic liver transplantation. J Clin Invest. 2007; 117 (9):2583–91. https://doi.org/10.1172/JCI31892 PMID: 17717604

67. Palmer LA, Gaston B. S-nitrosothiol assays that avoid the use of iodine. Methods in enzymology. 2008; 440:157–76. https://doi.org/10.1016/S0076-6879(07)00809-9 PMID: 18423216

68. Frostell C, Fratacci MD, Wain JC, Jones R, Zapol WM. Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary vasoconstriction. Circulation. 1991; 83(6):2038–47. https://doi.org/10.1161/01.cir.83.6.2038 PMID: 2040056

69. Hogman M, Frostell C, Arnberg H, Hedenstierna G. Bleeding time prolongation and NO inhalation. Lancet. 1993; 341(8861):1664–5.

70. Troncy E, Francoeur M, Salazkin I, Yang F, Charbonneau M, Leclerc G, et al. Extra-pulmonary effects of inhaled nitric oxide in swine with and without phenylephrine. Br J Anaesth. 1997; 79(5):631–40. https://doi.org/10.1093/bja/79.5.631 PMID: 9422904

71. Lee JS, Adrie C, Jacob HJ, Roberts JD Jr., Zapol WM, Bloch KD. Chronic inhalation of nitric oxide inhibits neointimal formation after balloon-induced arterial injury. Circ Res. 1996; 78(2):337–42. https://doi.org/10.1161/01.res.78.2.337 PMID: 8575078

72. Adrie C, Bloch KD, Moreno PR, Hurford WE, Guerrero JL, Holt R, et al. Inhaled nitric oxide increases coronary artery patency after thrombolysis. Circulation. 1996; 94(8):1919–26. https://doi.org/10.1161/01.cir.94.8.1919 PMID: 8873669

73. Hataishi R, Rodrigues AC, Neilan TG, Morgan JG, Buys E, Shiva S, et al. Inhaled nitric oxide decreases infarction size and improves left ventricular function in a murine model of myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol. 2006; 291(1):H379–84. https://doi.org/10.1152/ajpheart.01172.2005 PMID: 16443673

74. Fox-Robichaud A, Payne D, Hasan SU, Ostrovsky L, Fairhead T, Reinhardt P, et al. Inhaled NO as a viable antiadhesive therapy for ischemia/reperfusion injury of distal microvascular beds. J Clin Invest. 1998; 101(11):2497–505. https://doi.org/10.1172/JCI2736 PMID: 9616221

75. Mathru M, Huda R, Solanki DR, Hays S, Lang JD. Inhaled nitric oxide attenuates reperfusion inflammatory responses in humans. Anesthesiology. 2007; 106(2):275–82. https://doi.org/10.1097/00000542-200702000-00015 PMID: 17264721

76. Wink DA, Kasprzak KS, Maragos CM, Elespuru RK, Misra M, Dunams TM, et al. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science. 1991; 254(5034):1001–3. https://doi.org/10.1126/science.1948068 PMID: 1948068

77. Sonveaux P, Kaz AM, Snyder SA, Richardson RA, Cardenas-Navia LI, Braun RD, et al. Oxygen regulation of tumor perfusion by S-nitrosohemoglobin reveals a pressor activity of nitric oxide. Circulation research. 2005; 96(10):1119–26. PMID: 15879309

78. Lipton AJ, Johnson MA, Macdonald T, Lieberman MW, Gozal D, Gaston B. S-nitrosothiols signal the ventilatory response to hypoxia. Nature. 2001; 413(6852):171–4. https://doi.org/10.1038/35093117 PMID: 11557982

79. Palmer LA, Doctor A, Chhabra P, Sheram ML, Laubach VE, Karlinsey MZ, et al. S-nitrosothiols signal hypoxia-mimetic vascular pathology. The Journal of clinical investigation. 2007; 117(9):2592–601. https://doi.org/10.1172/JCI29444 PMID: 17786245

80. Doctor A, Platt R, Sheram ML, Eischeid A, McMahon T, Maxey T, et al. Hemoglobin conformation couples erythrocyte S-nitrosothiol content to O2 gradients. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102(16):5709–14. https://doi.org/10.1073/pnas.0407490102 PMID: 15824313

81. Singel DJ, Stamler JS. Blood traffic control. Nature. 2004; 430(6997):297. https://doi.org/10.1038/430297a PMID: 15254518

82. Gaston B, May WJ, Sullivan S, Yemen S, Marozkina NV, Palmer LA, et al. Essential role of hemoglobin beta-93-cysteine in posthypoxia facilitation of breathing in conscious mice. J Appl Physiol (1985). 2014; 116(10):1290–9.

83. Palmer LA, May WJ, deRonde K, Brown-Steinke K, Bates JN, Gaston B, et al. Ventilatory responses during and following exposure to a hypoxic challenge in conscious mice deficient or null in S-nitrosoglutathione reductase. Respir Physiol Neurobiol. 2013; 185(3):571–81. https://doi.org/10.1016/j.resp.2012.11.009 PMID: 23183419

84. Roustit M, Cracowski JL. Non-invasive assessment of skin microvascular function in humans: an insight into methods. Microcirculation. 2012; 19(1):47–64. https://doi.org/10.1111/j.1549-8719.2011.00129.x PMID: 21883640

85. Turner J, Belch JJ, Khan F. Current concepts in assessment of microvascular endothelial function using laser Doppler imaging and iontophoresis. Trends in cardiovascular medicine. 2008; 18(4):109–16. https://doi.org/10.1016/j.tcm.2008.02.001 PMID: 18555183

86. Kelm M, Preik-Steinhoff H, Preik M, Strauer BE. Serum nitrite sensitively reflects endothelial NO formation in human forearm vasculature: evidence for biochemical assessment of the endothelial L-arginine-NO pathway. Cardiovasc Res. 1999; 41(3):765–72. https://doi.org/10.1016/s0008-6363(98)00259-4 PMID: 10435049

87. Gaston B, Reilly J, Drazen JM, Fackler J, Ramdev P, Arnelle D, et al. Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. Proceedings of the National Academy of Sciences of the United States of America. 1993; 90(23):10957–61. https://doi.org/10.1073/pnas.90.23.10957 PMID: 8248198

88. Moya MP, Gow AJ, McMahon TJ, Toone EJ, Cheifetz IM, Goldberg RN, et al. S-nitrosothiol repletion by an inhaled gas regulates pulmonary function. Proceedings of the National Academy of Sciences of the United States of America. 2001; 98(10):5792–7. https://doi.org/10.1073/pnas.091109498 PMID: 11320213