Nitric Oxide Synthesis in the Lung

Regulation by Oxygen through a Kinetic Mechanism

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Abstract

In this study, we show that oxygen regulates nitric oxide (NO) levels through effects on NO synthase (NOS) enzyme kinetics. Initially, NO synthesis in the static lung was measured in bronchiolar gases during an expiratory breath-hold in normal individuals. NO accumulated exponentially to a plateau, indicating balance between NO production and consumption in the lung. Detection of NO_2^-, NO_3^-, and *S*-nitrosothiols in lung epithelial lining fluids confirmed NO consumption by chemical reactions in the lung. Interestingly, alveolar gas NO (estimated from bronchiolar gases at end-expiration) was near zero, suggesting NO in exhaled gases is not derived from circulatory/systemic sources. Dynamic NO levels during tidal breathing in different airway regions (mouth, trachea, bronchus, and bronchiole) were similar. However, in individuals breathing varying levels of inspired oxygen, dynamic NO levels were notably dependent on O_2 concentration in the hypoxic range (K_mO_2 190 μM). Purified NOS type II enzyme activity in vitro was similarly dependent on molecular oxygen levels (K_mO_2 135 μM), revealing a means by which oxygen concentration affects NO levels in vivo. Based upon these results, we propose that NOS II is a mediator of the vascular response to oxygen in the lung, because its K_mO_2 allows generation of NO in proportion to the inspired oxygen concentration throughout the physiologic range. (*J. Clin. Invest.* 1998. 101:660–666.) Key words: nitric oxide • nitric oxide synthase • oxygen • ventilation-perfusion ratio • kinetics

Introduction

Oxygen is the major physiologic regulator of ventilation perfusion matching in the lung, through vasoconstriction of pulmonary vessels in regions of low ventilation containing low oxygen levels (1–7). The mechanisms by which oxygen regulates pulmonary vascular tone are not completely understood, but several studies have suggested that nitric oxide (NO)[1] plays a central role in oxygen-induced vasodilatation (1–7). NO is synthesized endogenously by NO synthases (NOSs; EC 1.14.13.39), which convert L-arginine to L-citrulline and NO in the presence of oxygen, NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin, and calmodulin (8). Three NOSs (types I, II, and III) have been identified in the human lung (9, 10). Type I and III NOS are expressed primarily in neuronal and endothelial cells of the normal human lung, respectively, and are dependent on increases in intracellular calcium for enzyme activation and picomolar levels of NO production (8–10). Type II NOS is the major NOS protein expressed in normal human airway epithelium, is calcium independent, and produces nanomolar levels of NO (8–11). NO production has also been identified in the paranasal sinuses, although the NOS enzyme present in the upper respiratory tract is not known (12, 13).

Once produced, NO is freely diffusible, and may enter pulmonary smooth muscle cells to activate soluble guanylate cyclase and produce cGMP (6, 14–16). Increased cGMP in pulmonary vascular smooth muscle cells activates a cGMP-sensitive kinase which phosphorylates a calcium-dependent potassium channel leading to hyperpolarization and vasodilatation (6, 14–16). Whereas animal studies point to the possibility that NO is the intracellular mediator of oxygen's vasoregulatory properties (2, 4–6), studies in humans demonstrating that endogenous NO levels or NOS activity vary quickly and in direct proportion to oxygen levels are lacking. Further, the mechanisms by which oxygen may rapidly modulate NO levels/activity are unclear. Potential mechanisms for hypoxia quickly decreasing NO include rapid inactivation/scavenging of NO, decreased NOS enzyme activity, or decreased substrate/cofactor availability. In this study, the kinetics of NO synthesis in the lung and its regulation by oxygen were investigated in normal individuals. Our results show that gas phase NO accumulation in the static human lung occurs as a first-order process, resulting in a steady state NO level in vivo, and that NO levels decrease as inspired oxygen levels decrease below ambient air. One mechanism to explain oxygen's effect on NO levels in the lung is revealed by enzyme kinetic analysis, which demonstrates that molecular oxygen concentrations in the physiologic range determine the rate of NO synthesis by purified NOS II, the major airway epithelial isoform (11).

Methods

Intrapulmonary NO levels. Normal, nonsmoking individuals (nine men and two women, 34±2 yr) underwent flexible fiberoptic bron-

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1. *Abbreviations used in this paper:* AUC, area under the curve; BAL, brochoalveolar lavage; ELF, epithelial lining fluid; FAD, flavin adenine dinucleotide; FiO_2, fraction of inspired oxygen; FMN, flavin mononucleotide; NO, nitric oxide; NOS, NO synthase.

choscopy under local anesthesia as described previously (11). The bronchoscope was advanced into the lung, and real time NO measurements were obtained at a rate of 20 samplings/s using a Teflon tube inserted through the working channel of the bronchoscope and connected to a chemiluminescence analyzer for detection of nitric oxide (NOA 280; Sievers, Boulder, CO), which was calibrated daily using NO-free gas (zero NO) and 8.7 parts per million (ppm) NO gas (12). The dead-time efficiency for NO measurement in the NOA 280 was determined to be $<$ 90 ms. Individuals were instructed to perform normal tidal breathing or breath-holds during NO measures. Breath-holding was confirmed by monitoring chest wall movement and airflow at the mouth and nose. Intrapulmonary NO levels were measured on individuals breathing room air, and while breathing air with no NO (zero air) when ambient levels were $\geq$ 20 parts per billion (ppb).

Measurement of NO_2^-, NO_3^-, and S-nitrosothiols in bronchoalveolar lavage (BAL) fluid. BAL was performed as described previously (11). Briefly, three 50-ml aliquots of sterile normal saline solution were infused into a segmental or subsegmental bronchus and then aspirated back. Nitrate, nitrite, and *S*-nitrosothiols present in BAL fluid were converted to NO by a saturated solution of VCl_3 in 0.8 M HCl, and the NO was detected based on a gas phase chemiluminescent reaction between NO and ozone using the NOA 280 (17). Nitrite alone was determined using a 1% w/vol solution of KI in glacial acetic acid to convert NO_2^- to NO, and NO was detected by chemiluminescence as above (17). Nitrite and nitrate standards were also tested. Nitrite and nitrate standards displayed linearity between 0.07 and 10 μM ($r^2 \geq 0.90$ for all experiments) and were detected with equal efficiency ($<$ 15% difference in detection at any concentration). NO levels were determined by interpolation from the known standard curves. Levels of NO_2^-/NO_3^-/*S*-nitrosothiol were expressed as micromolar levels in epithelial lining fluid (ELF). The volume of ELF recovered by BAL was quantified by the urea method (18). Urea concentrations in BAL and in plasma were determined with the BUN 20 endpoint assay (Sigma Chemical Co., St. Louis, MO).

Exhaled NO levels. Levels of exhaled NO were determined over the course of normal tidal breathing by measuring NO in exhaled gases at 20 times/s using the NOA 280. Normal, nonsmoking individuals (nine men and two women, 33±4 yr) were instructed to perform tidal breathing through a tight-fitting face mask, while wearing nose clips. The face mask was connected in line with the NOA 280 to measure NO levels, and with the Prospector exercise system (Cybermedic, Louisville, CO) to measure CO_2 and O_2 (19, 20). The Prospector exercise system contains a zirconium electrochemical cell for determination of oxygen and an infrared analyzer for carbon dioxide, and has precision of ±0.5% for O_2 and CO_2 (19, 20). NO, O_2, and CO_2 were measured while individuals were breathing varying fractions of inspired oxygen (FiO_2) (0.50, 0.30, 0.21, 0.15, 0.10, and 0.05 FiO_2). Oxygen mixtures were prepared the same day by mixing O_2 and N_2 in a nondiffusing gas-collection 60-liter bag (Hans Rudolph Inc., Kansas City, MO), with O_2 concentrations verified by the O_2 zirconium electrochemical cell in the Prospector exercise system. Oxygen mixtures contained $<$ 0.5 ppb NO as measured by the NOA 280. Individuals breathed each oxygen mixture for a minimum of 15 s to allow for equilibration of O_2 levels in the lung. After the equilibration period, NO levels during tidal breathing were measured for 60 s (15–30 s for volunteers breathing 0.05 FiO_2, depending on volunteer tolerance). The study was approved by the Cleveland Clinic Foundation Institutional Review Board, and written informed consent was obtained from all individuals.

NOS enzyme kinetics. Concentrated type II NOS (8.7 μg) was placed in septum-sealed cuvettes and mixed with various ratios of N_2-, air-, or O_2-saturated buffer solutions that contained 40 mM BisTris propane, pH 7.4, 1 mM L-arginine, 0.3 mM DTT, and 4 μM each of FMN, FAD, and tetrahydrobiopterin (1 ml final vol). Type II NOS was generated from cell lysates of *Escherichia coli* transformed with a plasmid containing murine NOS II, and purified by sequential metal chelate and 2′, 5′ ADP affinity chromatography as described previously (21). The NOS II recovered by this method is dimeric, contains normal quantities of heme, flavins, and bound calmodulin, and has high NO synthesis activity (21). The initial O_2 concentration in each reaction was calculated based on the solution mixing ratio and the O_2 concentration of air (21%)-saturated or 100% O_2-saturated buffer at 25°C (0.26 and 1.26 mM oxygen, respectively). Reactions were initiated by injection of 10 μl of NADPH (200 μM final concentration) and run at 25°C. The initial rates of NADPH oxidation were determined at 340 nm in the presence of L-arginine (22). K_mO_2 values were estimated from double reciprocal plots of the data.

Statistical analyses. NO levels in gases during tidal breathing were analyzed using a Microsoft Excel 4.0–based macro which calculates the peak, end tidal, area under the curve (AUC), and average NO levels for each breath. The peak NO level is the maximum level of NO in the exhalation, the end tidal value is the average NO level from 1/2 to 7/8 of the exhalation, the AUC is an integration of the area under the NO graph over the duration of each exhalation, and the average value is the mean level of NO over the whole exhalation. Exponential equation fit of data was performed using the SigmaPlot statistical program (version 3.0; Jandel Scientific, San Rafael, CA). Continuous variables were summarized by group as sample size, mean, and SEM. Statistical comparisons were performed using ANOVA. Linear correlation coefficients were estimated by Pearson's technique.

Results

NO in the respiratory tract. NO levels were measured at the mouth, just below the vocal cords, at the carina, the midportion of the mainstem bronchus, and at bronchioles between 5 and 7 mm in diameter. Variation in NO levels was noted with tidal breathing at all areas. In the intrathoracic airway, peak NO levels with inspiration were related to nasal production and could be excluded by oral breathing (Fig. 1 *A*). Ambient air NO levels also contributed slightly, but were not responsible for inspiratory peak levels of NO detected in the lower airway, which were similar in individuals breathing "zero" air (0 ppb NO) or ambient air (ambient air was measured daily and varied between 0 and 30 ppb NO). Thus, during normal nasal tidal breathing, there was pulsatile inspiratory delivery of NO to the lungs related to nasal production of NO (12, 13). NO peak, end tidal, average, and AUC values during oral breathing were determined for each area of the airway, and were correlated to each other (correlations of NO values: peak vs. average, $r = 0.966$; peak vs. AUC, $r = 0.734$; average vs. AUC, $r = 0.744$; AUC vs. end tidal, $r = 0.739$; peak vs. end tidal, $r = 0.969$; average vs. end tidal, $r = 0.991$). Results are shown for average NO levels. NO levels in each area of the airway during oral breathing were similar to levels measured at the mouth during oral breathing (*P*, NS; $n = 7$; Fig. 1 *B*). Interestingly, NO levels measured in the bronchioles decreased rapidly at end-exhalation in all individuals, measuring between 0 and 2 (1.1±0.35) ppb. The near zero NO levels in end-exhalation gases are likely a reflection of alveolar gas NO levels, since hemoglobin has an extremely high affinity for NO (about 3,000 times that of oxygen) and would scavenge NO from alveolar gases (14). This finding supports previous reports that NO present in exhaled gases does not reflect delivery from the circulation or from systemic sites (23, 24).

The consumption of NO by hemoglobin from the alveolar gases in combination with breath-hold maneuvers was used in order to measure more accurately the rate of NO accumulation in the airway (bronchioles 5–7 mm in diameter) in the absence of airflow and nasal NO production. Individuals were in-

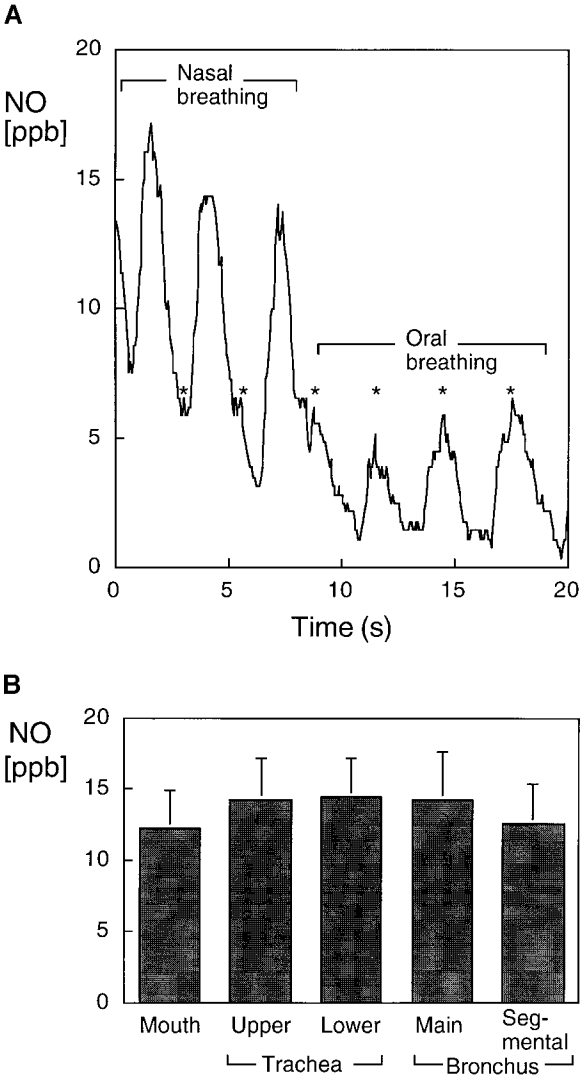

Figure 1. (*A*) NO levels in a bronchiole of the human lung with nasal and oral breathing. NO was measured in a bronchiole via bronchoscopy in a normal individual during tidal breathing. A sampling rate of 20/s allowed for real time observation of NO over the course of normal tidal breathing. During nasal breathing, peak NO levels occurred with inspiration, likely related to inhalation of NO produced in the paranasal sinuses. When the individual switched to oral breathing, peak NO levels occurred during exhalation. During nasal breathing, expiratory NO peaks (*) can be seen buried within the exhalation phase. Nasal contribution to lower airway NO can be effectively excluded by oral breathing. (*B*) NO levels at different regions of the airway. NO was measured at the mouth, subglottic area (*Upper Trachea*), carina (*Lower Trachea*), main bronchus, and bronchiole (*Segmental Bronchus*). NO measured in all areas of the airway were similar ($n = 7$ individuals). Mouth measures of NO accurately reflect lower airway levels.

structed to inspire to total lung capacity and breath-hold (10 s) during bronchoscopy to allow for complete removal of NO by hemoglobin in the alveoli (generating NO-free gas distal to the bronchoscopic sampling catheter). Individuals were then instructed to exhale rapidly until NO-free gases from the alveoli reached the level of the sampling tube, ascertained by NO levels < 3 ppb at the level of the sampling catheter. At this point, individuals were instructed to breath-hold, and the accumulation of NO was recorded. In all individuals, NO accumulated rapidly, followed by a plateau (time for rapid accumulation phase of NO, range 10–15 s, 11.4±0.8 s, $n = 5$) (Fig. 2). Expiratory breath-hold times varied by individual (10–40 s, 22±5 s, $n = 5$); however, once achieved, the NO plateau was maintained over breath-hold times of up to 40 s (NO plateau, 7.1±0.9 ppb, $n = 5$). At the end of the expiratory breath-hold, individuals exhaled to residual volume, and NO levels again decreased rapidly to near 0 ppb at the sampling catheter in all individuals. Although bronchiolar gases demonstrated accumulation of NO over the time of expiratory breath-hold, alveolar gases did not accumulate NO. Thus, NO accumulation in the bronchiolar gases was due to local synthesis within the lung/airway, and was not related to circulatory delivery of NO with diffusion into the airway. The rate of NO accumulation in bronchiolar gases with expiratory breath-hold was fit with an exponential equation using the following formula:

$$\text{NO [ppb]} = 5.6 \times (1 - e^{(-0.25 \times t)}) + 2.0,$$

where t represents time in seconds, and e is 2.718. Using this curve fit, the equation describes NO accumulation in the gas phase as a single exponential process with a first-order rate constant of 0.25±0.06 s^{-1} ($n = 5$). These results confirm that NO synthesis occurs in the lung in isolation of airflow, and in exclusion of NO in ambient air and the upper respiratory tract.

Lung ELF NO products (NO_2^-, NO_3^-, and S-nitrosothiols). Based on the known partition coefficient of NO between the gas and liquid phase (20:1), NO in the gas phase could represent up to 95% of the NO present in the ELF and cells within the airways (see p. 735 in reference 25). However, NO produced by cells is freely diffusible in all directions, not just

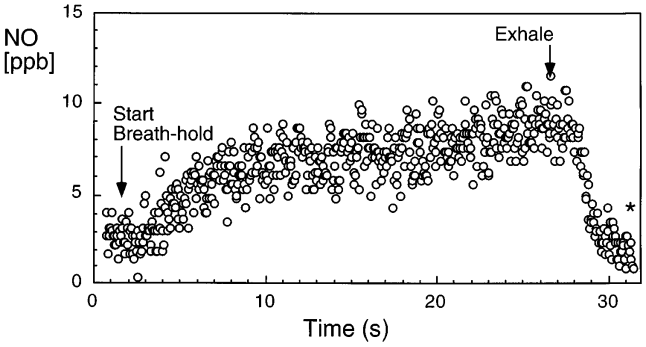

Figure 2. Kinetics of NO accumulation in the gas phase of a bronchiole in the static human lung. NO was measured continuously in the bronchiole during bronchoscopy of normal individuals ($n = 5$) during an expiratory breath-hold maneuver. A representative expiratory breath-hold from a normal volunteer is shown. After a deep inspiration to total lung capacity, with inspiratory breath-hold for 10 s (not shown), the volunteer exhaled until NO levels at the sampling catheter in the bronchiole approached zero (reflecting NO "free" alveolar gases reaching the level of the catheter). At this point, the individual started breath-hold (*arrow*) for as long as tolerated. During the expiratory breath-hold, bronchiolar gases accumulated NO quickly to a plateau, which could be predicted using a single exponential equation (see text). At the end of expiratory breath-hold, the individual exhaled (*arrow*) completely to residual volume, which caused NO levels to decrease rapidly. Near zero NO levels were observed at the end of exhalation (*), indicating that alveolar gases do not accumulate NO.

outward into the airway. Furthermore, NO undergoes chemical reaction with oxygen and superoxide to form oxidation products (NO_2^- and NO_3^-) and with biologic constituents to form *S*-nitrosothiols; 100% of these reaction products are partitioned in the liquid phase. Lung ELF has been shown previously to contain NO_2^- and *S*-nitrosothiols (26). NO reaction products (NO_2^-, NO_3^-, and *S*-nitrosothiols) were also detected at high levels in the ELF of normal individuals in this study (NO_2^- 8.5±2.6 μM in ELF, nitrosothiols and NO_3^- 166±46 μM in ELF; $n = 5$). In the context of the poor solubility of NO, nasal NO is unlikely to contribute significantly to formation of NO reaction products in the lower airway. These results suggest that lower airway NO detected in the gas phase during the breath-hold represents diffusion from a tissue source of NO in airway cells, but the gas phase NO levels likely represent only a fraction of the total NO production in the lung.

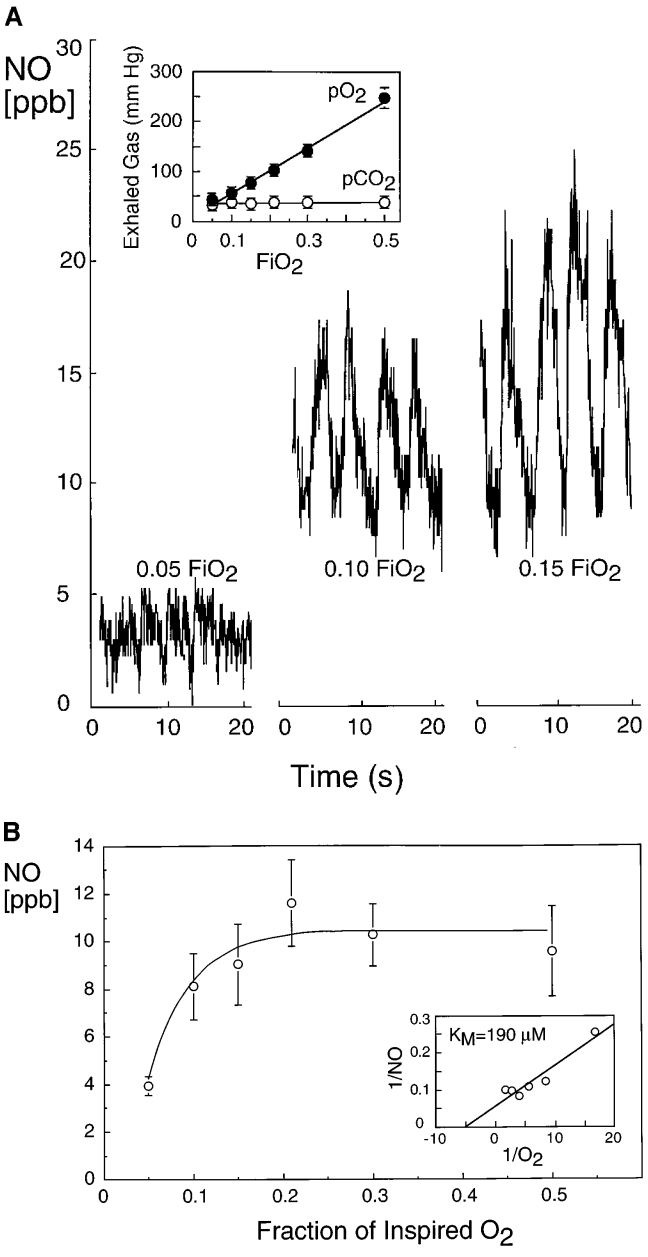

Figure 3. NO levels are dependent on inhaled oxygen levels. (*A*) NO measured at the mouth during tidal breathing from a representative individual with varying FiO_2. NO decreases with decreasing FiO_2. (*inset*) Exhaled O_2 and CO_2 levels with varying FiO_2 ($n = 3$–11 individuals for each). (*B*) Mean NO levels with varying FiO_2. NO is correlated with oxygen levels in the hypoxic range, decreasing as oxygen levels decrease below ambient air (FiO_2 0.21). (*inset*) Double reciprocal plot of the mean NO (in parts per billion) and inspired O_2 (millimolar) for determination of the K_mO_2 (190 μM).

Regulation of NO by oxygen in vivo. Similar to levels in the lower airway, breath by breath analyses of NO at the mouth demonstrated variation in NO levels during oral tidal breathing. NO levels peaked early in exhalation, with decreasing levels of NO at end-exhalation. Since levels of NO at the mouth were similar to lower airway levels (Fig. 1 *B*), NO levels were measured at the mouth for individuals ($n = 11$) breathing varying FiO_2. Due to differing abilities of individuals to tolerate varying oxygen levels, not all individuals inspired each oxygen level (FiO_2 0.10, 0.15, and 0.21, $n = 11$ each; FiO_2 0.30 and 0.50, $n = 8$ each; FiO_2 0.05, $n = 3$). To assure that individuals were actually receiving the desired FiO_2, exhaled O_2 levels were measured and were highly correlated with inhaled FiO_2 (correlation of inhaled to exhaled oxygen levels, $r = 0.991$, $P < 0.05$; Fig. 3 *A*, *inset*). Exhaled CO_2 levels decreased slightly with decreasing FiO_2 (FiO_2 0.05, CO_2 [mmHg] 34±2.8; FiO_2 0.10, CO_2 36.8±0.6; FiO_2 0.15, CO_2 35.6±1.8; FiO_2 0.21, CO_2 36.9±1.1; FiO_2 0.30, CO_2 39.2±0.8; FiO_2 0.50, CO_2 37.9±1.7; $r = 0.523$, $P < 0.05$; Fig. 3 *A*, *inset*). NO levels were dependent on inhaled oxygen levels (ANOVA, $P < 0.05$). Levels of NO were directly correlated to inspired oxygen levels in the hypoxic range ($FiO_2 < 0.21$) (Fig. 3, *A* and *B*). A decrease in NO levels was observed throughout the hypoxic range, but the greatest decrease in NO occurred in each of the three individuals breathing an FiO_2 of 0.05. Increasing inhaled O_2 above ambient (FiO_2 0.21) did not increase NO (Fig. 3 *B*). The apparent K_m for oxygen as calculated from a double reciprocal plot of the data was 190 μM (linear regression $r = 0.951$, Fig. 3 *B*, *inset*).

Dependence of NOS II kinetics on molecular oxygen in vitro. To investigate why NO concentrations in respired air decreased with decreasing O_2 levels in vivo, the rate of NO synthesis by purified NOS II was investigated with varying oxygen levels in vitro. We have shown previously that the airway epithelium expresses predominantly type II NOS, the high output NO synthase (11). Since NO accumulation within the lung was detected above the surface of the airway epithelium, regulation of NOS II by oxygen in the physiologic range was investigated. Similar to the in vivo dependence of NO production on oxygen, rates of NOS II NADPH oxidation (Fig. 4) associated with NO synthesis (not shown) increased in a near linear manner with O_2 concentration in the physiologic range (0–250 μM) and then plateaued at the higher range of O_2. The dependence on oxygen levels occurred despite saturating concentrations of cofactors and L-arginine substrate. The apparent K_mO_2 value estimated from a double reciprocal plot of the data was 135 μM (Fig. 4, *inset*).

Discussion

The primary finding of our study is that the NOS activity present in the lung is regulated by oxygen. Furthermore, in vitro experiments using purified NOS II identify a mechanism

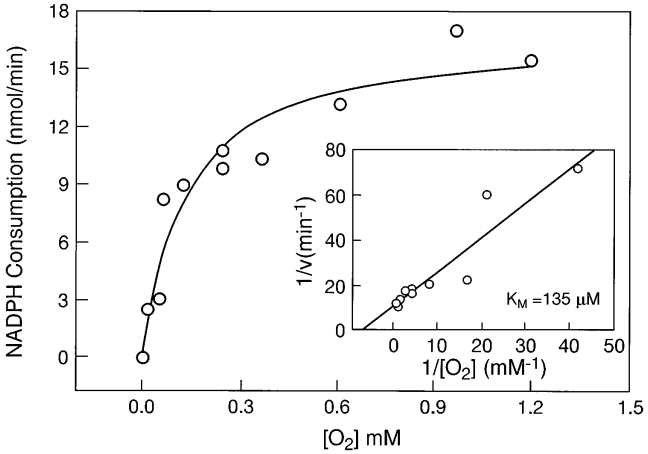

Figure 4. NOS II enzyme kinetics are dependent on oxygen concentration. Activity of purified NOS II as measured in vitro by NADPH oxidation is dependent on oxygen concentration in the physiologic range ($\leq$ 260 μM O_2). (*inset*) Double reciprocal plot of NADPH consumption and O_2 levels for determination of the NOS II K_mO_2 (135 μM).

operating at the enzyme level that can explain oxygen regulation of NOS activity in the lung.

Previous studies in rabbits have shown that NO detected in exhaled gases is derived primarily from the lung and not due to delivery from blood circulation, since NO levels in exhaled gases remained after blood circulation was stopped by intravenous injection of air or helium (23). Synthesis of NO in the human lung was confirmed in this study by measurement of NO accumulation in the bronchiolar gas phase in the absence of tidal ventilation. The accumulation of NO in bronchiolar gases was exponential, with an initial linear rise followed by a plateau. The steady state levels of NO were remarkably similar in all individuals, as were the first-order rate constants of NO accumulation. Interpretation of the steady state level of NO in the bronchiolar gas phase and its relevance to lung tissue levels of NO requires an understanding of the distribution of NO in tissues. The spatial distribution of NO in an organ such as the lung will be dependent on the solubility of NO (gas–liquid interfaces), diffusibility of NO, the rate at which NO is synthesized, and the rate at which NO is consumed by oxygen species, proteins, and scavengers of NO such as hemoglobin (27, 28). NO is an unusual biologic messenger in that its movements are dependent on free diffusion; thus, net movement of NO occurs from regions of higher to regions of lower concentration (27, 28). Theoretical modeling predicts that since NO has a rapid rate of diffusion (3,300 $\mu M^2/s$), steady state levels are spread out over large distances and attained quickly (27–29). Similar to the pattern we observed for NO accumulation in bronchiolar gas, in vitro quantitation of the rate of appearance of NO at different locations in aortic tissue from a single NO-producing cell source demonstrated that NO accumulated in a linear fashion over 13 to 20 s and then achieved a steady state plateau concentration (30). Thus, NO production is accurately reflected by measures at a distance from a point of synthesis (27–30). Similarly, a multicompartment model predicts that several NO-producing cells will lead to linearly increasing NO levels until a steady state is reached within 15–20 s (28). In this study, the steady state NO levels in the bronchiolar gases of the lung were achieved within 10–15 s. This initial increase measured in the bronchiolar gas phase likely represents the rapid partitioning by diffusion of NO into the gas phase from the ELF/cytosol of NO-producing cells in the airway.

Attainment of steady state levels indicates a constant rate of production balanced by a constant consumption or scavenging of NO (27–30). For example, in an in vitro system of a uniform layer of endothelial cells synthesizing NO at a constant rate, it has been determined that 37% of the NO synthesized is consumed by chemical reactions (30). Since NO is freely diffusible, consumption of NO can occur at different sites within the cell, lung tissue, extracellular fluids, and intravascular compartments. Primary reactions that may consume NO intra- and extracellularly include its reaction with oxygen, superoxide, hemoglobin, another molecule of NO, enzymes containing iron-sulfur centers, heme-containing proteins, and thiol proteins, and mitochondrial consumption (31–34). For example, NO diffusing to arterial myocytes will bind to the heme of guanylate cyclase, while NO diffusing to ELF may be consumed in the formation of the *S*-nitrosyl derivative of glutathione (26, 31, 33). During catalysis, NOS also binds self-generated NO, causing it to operate at a fraction of its maximum possible activity during the steady state (32, 35, 36). Although autoxidation of low levels of NO has been estimated to be a slow process (31, 37, 38), in the presence of lipid this process is accelerated up to 300-fold (J.R. Lancaster, Louisiana State University, personal communication). Thus, autoxidation may be particularly important in the lung, which contains a large surface area lined with fluid abundant in phospholipids, i.e., surfactant. In any case, measured levels of NO reaction products in the lung ELF indicate that a significant proportion of the NO produced is consumed by these processes, and that gas phase measures underestimate total NO produced within the lung.

An especially important scavenger of NO in the lung is hemoglobin (27, 31, 32, 39). NO produced in the lungs may diffuse into the lumen of blood vessels, where most will be trapped by hemoglobin in red blood cells (14, 27, 28, 39). Interestingly, oxygenated *S*-nitrosohemoglobin might play an important role in the regulation of systemic vascular tone (39). Hemoglobin through oxygenation-induced conformational changes also senses physiologic oxygen gradients and leads to changes in blood flow related to NO derivatives, in proportion to oxygen requirements (39). The addition of even very small amounts of hemoglobin has been shown to result in a substantial decrease in the steady state distribution of NO in vitro (27, 28). Considering the high diffusibility of NO, the extremely rapid rate of scavenging by hemoglobin, and the rich supply of blood vessels found in the lung, hemoglobin is undoubtedly a significant biologic sink for NO. Despite this, at least some of the endogenously produced NO diffuses into the airway and is eliminated via the gas phase. Thus, while NO levels in the gas phase likely underestimate NO levels in the lung tissue and at pulmonary vascular sites, the measured levels reflect in an accurate and qualitative manner the dynamics of NO production and consumption in the lung.

Regulation of NO synthesis by oxygen. The levels of NO detected in the airway were similar to levels detected at the mouth during oral breathing; thus, measurements of NO exhaled at the mouth accurately reflect lung tissue levels of NO. We found that changes in inspired oxygen concentration led to significant changes in exhaled NO. In contrast, a previous

study of exhaled NO levels in humans in the presence of 0.10 as compared with 0.21 FiO_2 did not demonstrate significant differences (40). This may have been due to the level of hypoxia used, or the method of measuring NO (a summation method for total NO over several breaths) (40). In this study, small changes in exhaled NO were found between 0.21 and 0.10 FiO_2, whereas larger changes occurred with 0.05 FiO_2. Similarly, NO levels in exhaled gases from lungs of anesthetized rabbits ventilated with hypoxic gas mixtures were reduced only modestly using 0.14 or 0.10 FiO_2, but decreased markedly using 0.06 FiO_2 (23). In addition, ventilation of an isolated neonatal pig lung with 0.075 FiO_2 led rapidly to decreased NO in exhaled gases and decreased NO_2^-/NO_3^- in the recirculating perfusate compared with ventilation with 0.21 FiO_2 (2). Thus, NO levels in the lung appear to depend largely on inspired oxygen levels.

Whereas long-term changes in NO production by hypoxia have been described, including transcriptional effects on NOS gene expression (41, 42), the rapid changes noted in this study indicate either an instantaneous change in NO production by NOS, or a change in NO consumption by scavengers. It is unlikely that rates of NO consumption by superoxide or hemoglobin would change rapidly or in proportion to varying inspired oxygen levels. Rather, a rapid change in NO production is likely, based on our observation that oxygen levels directly affect NOS II activity in vitro. Hypoxia causes changes in cellular respiration and metabolism that may affect cellular levels of cofactors required for NOS activity. Specifically, hypoxia reduces NADPH levels, with significant decreases in NADPH at oxygen levels < 15 μM (43–45). However, this is unlikely to be contributing to the observed decrease in NOS activity, since the lowest in vivo oxygen levels were > 60 μM, which do not affect NADPH availability. Further, purified NOS II in vitro exhibited similar dependence on O_2 despite excess concentrations of cofactors. All NOSs are comprised of an oxygenase domain that binds iron protoporphyrin IX (heme), tetrahydrobiopterin, and L-arginine, and a reductase domain that binds FMN, FAD, and calmodulin (8). The NOS heme iron participates in catalysis by binding oxygen and catalyzing the oxidation of L-arginine (22, 32, 35, 36). When NO is formed during catalysis, it can also bind to the heme iron in NOS I and II and form an inactive complex (22, 32, 35). Thus, oxygen and NO compete for the heme iron. In fact, the overall oxygen dependence of NOS I has been shown to depend on the rate of decay of the heme iron–NO complex, which is itself dependent on oxygen concentration (22). Although the K_mO_2 of NOS II (135 μM) determined here is lower than that of NOS I (400 μM), NOS II has also been shown to form heme iron–NO complexes, indicating that the mechanism of regulation by NO and oxygen is likely to be similar between the two enzymes (32). In contrast to the K_mO_2 value for NOS II described in this study, a K_mO_2 had been estimated previously at 6.3 μM (46). Differences in measurement techniques (continuous assay of NADPH oxidation as opposed to citrulline endpoint) or in the source of NOS II studied may be responsible for the variance. Interestingly, the K_mO_2 value we derived for NOS activity in human lungs is very similar to the K_mO_2 for purified NOS II, suggesting that NO levels measured in the gas phase of the lung are derived mostly from NOS II activity in that tissue.

The oxygen concentration in intact tissues ranges from 1 to 150 μM (47–49), with the highest levels found in the lung. Airway epithelial cells are unique in their exposure to oxygen, since above a thin layer of ELF, the airway cells are exposed directly to air containing 21% oxygen. Based on oxygen solubility and the low differential oxygen gradient between overlying fluid and intracellular endoplasmic reticulum (1–2 μM) (49), the levels of oxygen in airway epithelial cells may actually approach 260 μM. Thus, the K_mO_2 we determined for NOS II in vitro is well within the physiologic range of oxygen concentrations in lung epithelial cells, and predicts an important role for oxygen in directly regulating NOS II activity in vivo, such that a steady level of NO production is maintained with normoxia and hyperoxia, and decreasing NO production with hypoxia.

NO as a mediator of vascular response to oxygen. Studies in pulmonary endothelial cells, isolated pulmonary vascular rings, isolated perfused lungs, and whole animals support an important role for NO in modulating the pulmonary vascular response to oxygen (1–7). The free diffusion of NO and the close apposition of airways to medium-sized pulmonary vessels which modulate pulmonary vessel tone (50) suggest that endogenous NO production in airways proximal to the alveolus may mediate pulmonary vasodilatation. Furthermore, hemoglobin in blood vessels may serve as a natural biologic sink for NO, creating a continuous concentration gradient for NO to move toward perivascular myocytes and thus regulate blood flow. Our findings support a mechanism in which NOS II mediates vascular response to oxygen in the lung by generating the vasodilator NO at a rate that is proportional to the inspired oxygen concentration throughout the physiologic range.

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