Medical Education Systems, Inc.

Inhaled Nitric Oxide Therapy

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Inhaled Nitric Oxide Therapy

Course Number: 981207 **3 CEUs**

Learning Objectives

Upon completion of this module, you will be able to:

- Explain what nitric oxide is and why healthcare professionals are so interested.
- Identify the physical and biological properties of nitric oxide.
- Summarize research findings regarding use of nitric oxide therapy in animals and humans.
- Identify and discuss the three major areas of concern regarding the toxicity of nitric oxide.
- Identify and explain the effects of nitric oxide therapy.
- List and explain the clinical indications for inhaled nitric oxide therapy.
- Identify monitoring systems and procedures associated with nitric oxide therapy.

Overview

What is Nitric Oxide?

Nitric oxide is a colorless, nonflammable and toxic gas which supports combustion. Nitric oxide is produced by oxidation of ammonia at high temperatures in the presence of a catalyst. In combination with air, nitric oxide forms brown fumes of nitrogen dioxide (NO_2). Together, these two gases are strong respiratory irritants which can cause chemical pneumonitis and a fatal form of pulmonary edema. Exposure to high concentrations of nitric oxide alone can cause methemoglobinemia.

Table 1.
Physical and Biochemical Properties of Nitric Oxide

High diffusibility
High lipid solubility
Gas or solution at body temperature
Reactive free radical
Short half-life (3-50 s)
Oxidized to NO_2 in air
Oxidized to nitrites and nitrates in solutionmilnes and n
Activity inhibited by hemoglobin, myoglobin, methylene blue, superoxide
Activity potentiated by superoxide dismutase, cyiochrome C, hydrogen ions

Nitric oxide is one of the most potent substances released by the vascular endothelium, and continuous formation acts on underlying smooth muscle to maintain vasodilation and blood flow. NO is a neuronal mediator that may be involved in neurotransmitter release, and synthesis of NO in the CNS involves the same pathways and same enzyme as in the endothelium. It now appears that stimulation of the glutamate receptor during ischemic reperfusion causes a prolonged release of NO, with subsequent tissue damage. Thus NO in the brain can be both beneficial by protecting, enhancing, and mediating the activity of neurons; and toxic by indiscriminately destroying neurons.

NO is found in nerves of the gastrointestinal and urogenital systems where it is involved in GI peristalsis and penile erection (you have of course heard all the raves about Viagra). It also plays a role in the immune system, which when activated results in the induction of macrophage NO synthase, which generates large amounts of NO from L-arginine.

Researchers have also found that nitric oxide is present in the exhaled gas of both humans and animals. While the research findings vary widely, most report finding exhaled NO <50 parts per billion (ppb) in normal persons. The quantity of exhaled NO is generally found to be higher in asthmatics or persons with bronchiectasis, but is generally lower in hypertensive persons and those who smoke. Animal studies have shown that exhaled NO is higher with the administration of positive end-expiratory pressure (PEEP), sepsis, sodium nitro prusside, or nitroglycerin. While the source of endogenous nitric oxide is not known with certainty, researchers speculate that the origin of NO in the exhaled

gas could either be the upper airway, the lower air-ways, or the lung parenchyma. They also speculate that exhaled NO could be the result of either cNOS or iNOS activity.

Studies show that a considerable amount of the NO in gas exhaled by normal persons is from the upper respiratory tract. Since nasopharyngeal bacteria stimulate the nasal mucosa to produce NO, anti-microbial therapies may result in lower NO production in the nasopharynx.

Researchers also suggest that the bacteriostatic effects of NO may be responsible for maintaining the sterility of the sinuses, since it has been shown that NO levels are relatively high in the paranasal sinuses.

Autoinhalation of NO produced in the nasopharynx may also be important to maintenance of the ventilation-perfusion relationship within the lung. Some clinical researchers have shown that autoinhalation of NO is reduced with antibiotic therapy, and others suggest that low-dose inhaled NO (100 ppb) in ARDS patients might be considered replacement therapy since the autoinhalation of nasopharyngeal NO is eliminated by endotracheal intubation.

Inhaled nitric oxide might help reduce breathing failure in preterm babies.

Breathing failure in premature newborn babies can be complicated by lung hypertension (raised blood pressure in the lungs). Sedation, muscle relaxation or ventilation (mechanically assisted breathing) are used to treat lung hypertension. Nitric oxide is believed to help regulate muscle tone in the arteries of the lungs but it could also cause excessive bleeding (hemorrhage). The review of trials found nitric oxide therapy probably does not improve the chances of the baby having an improved outcome, but also probably did not have adverse effects on the developing lung, and did not increase bleeding.

Background

Inhaled nitric oxide has been proven effective in term infants with hypoxic respiratory failure. The pathophysiology of respiratory failure, and the potential risks, differ substantially in preterm infants. Analysis of the efficacy and toxicities of inhaled nitric oxide in infants born before 35 weeks is therefore necessary.

Objectives
To determine whether, in preterm newborn infants (< 35 weeks gestation) who have hypoxic respiratory failure, treatment with inhaled nitric oxide improves oxygenation within 2 hours and reduces the rates of death, bronchopulmonary dysplasia, intraventricular hemorrhage, or neurodevelopmental disability

Search strategy
Standard methods of the Cochrane Neonatal Review Group were used. We searched MEDLINE, EMBASE, Healthstar and the Cochrane Central Register of Controlled Trials (CENTRAL, The Cochrane Library), using the following keywords: nitric oxide, clinical trial, newborn, and covering years from 1985 to 2005. In addition, we searched the abstracts of the Pediatric Academic Societies.

Selection criteria
- Randomized and quasi randomized studies in preterm infants with hypoxic respiratory failure.
- Administration of inhaled nitric oxide compared to control with or without placebo.
- Clinically relevant outcomes that were analyzed included death, bronchopulmonary dysplasia (defined as oxygen dependence at 36 weeks postconceptional age), intraventricular hemorrhage, long term neurodevelopmental outcome and short term effects on oxygenation.

Data collection and analysis
Standard methods of the Cochrane Neonatal Review Group were used. Two investigators extracted, assessed and coded separately all data for each study. Any disagreement was resolved by discussion.

Main results
Seven randomized controlled trials of inhaled nitric oxide therapy in preterm infants were found. One study consisted of infants determined to have a high risk of developing bronchopulmonary dysplasia (Subhedar 1997). One study studied routine use of inhaled NO in all ventilated preterm infants (Schreiber 2003). The remaining studies consisted of infants with high predicted mortality based on poor oxygenation (Kinsella 1999; Hascoet 2005; INNOVO 2005; Van Meurs 2005; Mercier 1999).

No significant effect of inhaled nitric oxide on mortality or bronchopulmonary dysplasia was demonstrated. There was no evidence of effect on the risk of intraventricular hemorrhage. There may be short-term improvements in oxygenation.

Two studies (Schreiber 2003; INNOVO 2005) have so far presented data

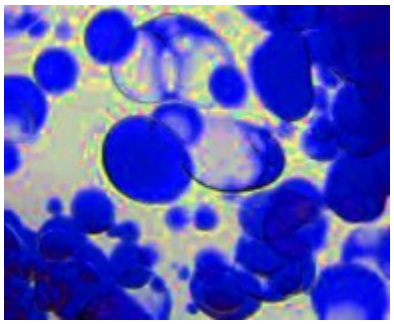
on long term neurodevelopmental outcome, one of which demonstrated improved outcome at two years corrected age.

Authors' conclusions
The currently published evidence from randomized trials does not support the use of inhaled nitric oxide in preterm infants with hypoxic respiratory failure.

Further studies may need to be performed to evaluate the potential benefit of routine use of this therapy in infants with milder forms of respiratory failure, and these trials will need to be designed to evaluate not only neonatal survival, and the occurrence of neonatal morbidities, but should be powered to evaluate neurodevelopmental outcome at a minimum of two years of age.

Pharmacology of Nitric Oxide

Elaborating on the answer to "what is nitric oxide?", we should review its pharmacology. As discussed earlier, nitric oxide is formed endogenously in vascular endothelial cells of the respiratory tract, is the active form of nitrovasodilators such as nitroglycerin and sodium nitroprusside, and has also been identified as at least one of the neurotransmitters in the nonadrenergic, non-cholinergic (NANC) inhibitory nervous system.



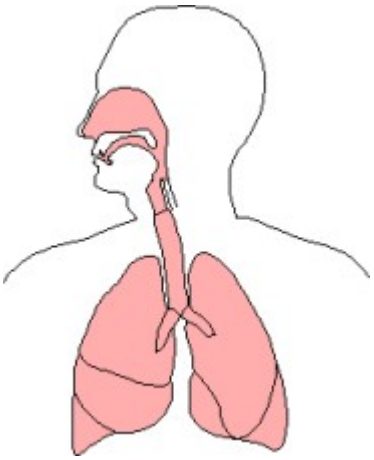
NO is formed from the precursor amino acid L-arginine by several isoforms of the enzyme, nitric oxide synthase, or NOS. Nitric oxide is generated in vascular endothelial cells, diffuses rapidly into myocytes in the endothelium, binding to guanylyl cyclase which stimulates the production of cyclic guanosine(c) 3',5'-monophosph-ate (cGMP), causing a reduction in intracellular calcium and consequent vascular or nonvascular smooth muscle relaxation.

The increase in cGMP within the cells, induced by NO, also inhibits platelet adherence and aggregation. Nitric oxide: readily diffuses into the endothelial cells and blood vessel itself, enters the red blood cell to bind rapidly with hemoglobin, forming methemoglobin, and becoming inactivated in the process.

In the red blood cells, nitric oxide is also converted to nitrate, with some endogenous NO being exhaled from the lung. Nitric oxide's action is limited to the pulmonary vascular endothelium, whether generated endogenously within the lung or inhaled as an exogenous gas, because it diffuses so readily into the blood stream and is inactivated by being bound to hemoglobin. This makes inhaled nitric oxide a *selective pulmonary vasodilator*.

The Biology of Nitric Oxide

Endothelium is essential for the vasodilator action of acetylcholine in isolated strips of arteries, and stimulation of the endothelial cells results in the release of endothelium-derived relaxing factor (EDRF). In the late 1980s, researchers found that nitric oxide accounted for the biologic activity of EDRF, and that L-arginine was its precursor.



Subsequently, researchers have found that nearly every cell type has the capacity to synthesize NO. NO has been shown to activate vascular smooth muscle relaxation, airway smooth muscle relaxation, neurotransmission, bacteriostasis, tumor-cell lysis, and platelet inhibition.

The substrate for NO synthesis in biologic systems is L-arginine. When NO-synthase is present, NO is produced and L-citrulline is formed as a by-product. NO serves as a local messenger molecule since it is highly lipophilic, and readily diffuses across cell membranes to adjacent cells. NO binds with guanylate cyclase once it has diffused into a nearby cell. Activation of guanylate cyclase causes production of cyclic guanosine 3',5'-monophosphate (cGMP) from guanosine triphosphate (GTP), which produces a biologic effect (like smooth muscle relaxation) within the cell.

Very little time elapses between NO production and guanylate cyclase activation, with a half life of <5 s for NO in physiologic systems. Inhibitors of guanylate cyclase (e.g., methylene blue) and inhibitors of NO-synthase reduce cGMP levels, while inhibitors of phosphodiesterase (e.g., zaprinast) increase cGMP levels.

Classifications of Isoforms of NO-synthase include: as constitutive NO-synthase (cNOS) or inducible NO-synthase (iNOS)--see Table 2. While cNOS is always present within cells, iNOS is expressed only after induction by a number of stimuli, including cytokines, microbes, or microbial products. There are two forms of cNOS: the endothelial type (eNOS), and the neuronal type (bNOS). Inhibitors of NO-synthase include L-N^G-monomethyl arginine (L-NMMA) and L-N^G-arginine methyl ester (L-NAME).

There are numerous research studies focusing on: learning more about the role NO plays in endotoxemia and sepsis, and discovering whether inhibition of iNOS in sepsis is beneficial or detrimental. Some findings have shown that NO-synthase inhibitors reverse hypotension during sepsis, but it is unknown if this action has any clinical importance.

Table 2.
Comparison of Constitutive (cNOS) & Inducible (iNOS)

Forms of Nitric Oxide Synthase
cNOS

- Cellular sources: endothelial cell, some neurons, mast cells, platelets Ca++ calmodulin dependent
- Transiently released in small amounts
- Activators: thrombin, acetylcholine, ADP and ATP, pressure and shear stress, bradykinin and histamine, leukotrienes
- Function is regulation (messenger molecule) iNOS
- Cellular sources: macrophages, hepatocytes, tumor cells, endothelial cells Ca++ calmodulin independent
- Sustained release in large amounts
- Inducers: lipopolysaccharide, interleukins, tumor-necrosis factor
- Inhibited by glucocorticoids
- Function is host defense

Some of the other biological aspects of nitric oxide include:

- NO has cGAP-independent efffects
- The cGNT-independent effects of NO may include bacteriostasis and tumor-cell lysis.
- NO reacts with transition metal ions and with both heme and non-heme metalloproteins.

- NO also reacts with a variety of other biologic metalloproteins including myoglobin and cytochrome oxidase.
- NO combines with O_2 to form nitrogen dioxide (NO_2) and with superoxide (O_2-) to form peroxynitrite.
- Potential by-products of NO metabolism include nitrite (NO_2-) and nitrate (NO_3-).

Research Studies

As mentioned earlier, nitric oxide's potential therepeutic effects have been the subject of a great number of research and clinical trials. The scope of this CEU does not allow for a complete review of the research findings, but the following discussion of both animal and human studies should give you a good idea of the direction those studies have taken and the results they have provided.

Animal Studies

In the laboratory setting, animal models with ARDS have been treated with inhaled NO and most test subjects demonstrated improvement in pulmonary shunt and PVR, increased nitrite and nitrate levels, and higher levels of circulating cGMP 15. However, it needs to be noted that the animal studies used higher dosage levels of NO than subsequently used in human clinical trials with inhaled NO.

Following induced lung injuries (such as oleic acid injury, smoke inhalation, and sepsis), animals have been treated with inhaled NO and then had the redistribution of blood flow and V/Q evaluated using the multiple inert-gas-elimination technique (MIGET). This effect was demonstrated with oleic acid injury, smoke inhalation injury, and sepsis. In general, inhaled NO alone produced no significant improvement in PaO_2 or V/Q as evaluated by MIGET.

However, in one study, researchers found that after inducing acute lung injury with oleic acid there was a significant interaction between continuous positive airway pressure (CPAP) and inhaled nitric oxide. Following application of 10 cm H_2O CPAP, administration of inhaled NO resulted in an improvement in PaO_2. The possible implication of

these results may be that employment of conventional therapies like PEEP or mean airway pressure should be maximized prior to the administration of inhaled nitric oxide.

Other animal studies looking at potential therapeutic effects of inhaled nitric oxide have suggested that it may provide protection against oxidant-induced lung injuries, and that it has anti-inflammatory properties.

Research Studies

Human Studies

The use of inhaled nitric oxide in human subjects with ARDS has been the focus of numerous published studies. Rather than presenting a detailed and potentially confusing explanation of the widely varying results of those studies, the following identifies highlights from some of those studies' findings:

Researchers from the University of Chicago reported in the Nov. 27, 2003, issue of the New England Journal of Medicine, that low doses of inhaled nitric oxide can decrease the risk of chronic lung disease and death by nearly one-fourth in premature infants who have respiratory distress syndrome (RDS).

Nitric oxide also can cut the risk of severe bleeding into the brain and loss of brain tissue – devastating complications of prematurity – by almost half.
The combination of prematurity and RDS may be lethal despite aggressive treatment, including mechanical ventilation. Approximately 60,000 children are born each year in the United States weighing less than 1,500 grams (about 3.3 pounds). Many of those who survive suffer permanent lung damage, which can slow growth, increase susceptibility to infection and is associated with abnormal brain development.

In the study, 64 percent of infants who received standard therapy died or developed chronic lung disease, compared to only 49 percent of those who received standard therapy plus inhaled nitric oxide.

"Inhaled nitric oxide gives neonatologists a simple and effective tool to help protect premature infants," says Michael Schreiber, M.D., associate

professor of pediatrics at the University of Chicago and director of the study. "Our data demonstrate that starting nitric oxide soon after birth in at-risk babies has few downsides and makes a major difference in their long-term health."

Adding small amounts of nitric oxide to oxygen for neonates requiring ventilation may soon become standard practice, Schreiber predicts, stressing the need to start treatment early. "Chronic lung disease often begins in utero," he says, "so if you're going to intervene, you need to start therapy as soon as possible."

Inhaled Nitric Oxide Harmful for Lower Weight Preterm Infants

The conclusions of a Stanford study would recommend against the use of inhaled nitric oxide for very premature infants with profound respiratory failure, especially those who weigh 1,000 grams or less:

There appears to be a dividing line for premature babies who are helped by inhaled nitric oxide and those who are harmed. That line is a birth weight of 1,000 grams.

So it seems from a study published in the July 7, 2005 *New England Journal of Medicine.* A Stanford group found that inhaled nitric oxide appears to be harmful to infants weighing 1,000 grams or less with severe respiratory failure.

Infants who weighed 1,000 grams or less who were treated with inhaled nitric oxide had higher mortality (62% vs. 48% in the placebo group; P = 0.01) and a higher rate of severe intraventricular hemorrhage (43% vs. 33% in the placebo group; P = 0.03), said Krisa P. Van Meurs, M.D., of Stanford and colleagues.

However, infants weighing more than 1,000 grams had significantly lower rates of death or bronchopulmonary dysplasia than infants in the placebo group (50% vs. 69%; P = 0.03), Dr. Van Meurs and colleagues reported.

To some degree, these results contrasted with those in a study by Karen K.L. Mestan, M.D., and colleagues at the University of Chicago, also published in today's *NEJM*. In a previous issue of *NEJM*, they reported reduced rate of death or bronchopulmonary dysplasia in premature infants treated with nitric oxide, regardless of weight. In the current issue, they followed this report with one demonstrating improved neurodevelopmental outcomes at two years of age in the treatment group compared to the placebo group.

The most likely reason for the differing results is that the infants in Dr. Van Meurs's study were significantly more premature, smaller, and sicker than those in Dr. Mestan's study, said Richard J. Martin, M.B., F.R.A.C.P., and Michele C. Walsh, M.D., of Children's Hospital in Cleveland in an accompanying editorial. "Therefore, short-term use of inhaled nitric oxide cannot be considered an effective rescue therapy for very preterm infants with profound respiratory failure," they said. "In contrast, less ill preterm infants may benefit from this therapy, both in the short-term and over the long term, as suggested by the study by Mestan et al."

Dr. Van Muers's study randomly assigned 420 neonates with respiratory failure, born at less than 34 weeks gestation, with a birth weight of 401 to 1,500 grams, to receive either inhaled nitric oxide or placebo. The initial results were not encouraging. The rate of death or bronchopulmonary dysplasia was 80% in the treatment group compared with 82% in the placebo group ($P = 0.52$).

However, a post hoc analysis of subgroups according to birth weight revealed the dramatically different outcomes in infants weighing less than 1,000 grams compared with those who weighed more.

Dr. Mestan's study was a prospective, longitudinal follow up at two years of 138 children who had received either nitric oxide or placebo as premature infants with respiratory failure. Blinded examiners conducted neurologic examinations and neurodevelopmental assessments.

In the treatment group, 24% of the children had abnormal neurodevelopmental outcomes, compared with 46% of the placebo group (P = 0.01).

"By increasing the likelihood of a premature infant's survival without neurodevelopmental delay, inhaled nitric oxide may significantly improve the quality of life of both the child and his or her family," Dr. Mestan and colleagues concluded.

Nevertheless, further data are necessary for decision-making. As pointed out an editorial in NEJM on July 7, 2005, at least two more large, multicenter, randomized trials of prolonged inhaled nitric oxide exposure beginning shortly after birth are still under way. "Pending the results, it is prudent to avoid the use of inhaled nitric oxide in preterm infants in the first week of life. The benefits and risks of inhaled nitric oxide need further scrutiny before its use becomes widespread."

- Subjects lmprovement in the ratio of arterial oxygen tension to fractional concentration of inspired oxygen (PaO_2/FIO_2) and PAP with no change in systemic hemodynamics when NO was administered by inhalation at 20 ppm. Prolonged inhalation of NO did not result in tachyphylaxis.
- Onset of effect was rapid with initiation of inhaled NO, and the effect quickly stopped when NO was discontinued.
- Subjects with a mean PAP 30 mm Hg were more likely to respond to NO inhalation.
- Between 50-60% of patients with ARDS and septic shock failed to respond to inhaled NO. 17% of ARDS patients treated with inhaled NO failed to increase their PaO_2 by at least 10 mm Hg, and 37% of patients failed to decrease their mean PAP by at least 3 mm Hg.
- Some studies have shown small but significant improvements in VD/VT and PaO_2.
- Others have shown a small but clinically insignificant reduction in VD/VT and PaO_2 in patients who respond to inhaled NO.
- It is nearly impossible to predict which patients with ARDS will or will not respond to treatment with inhaled NO. Patient response may be related to the degree of alveolar recruitment.
- Since most studies have focused on evaluating the physiologic effects of inhaled NO on ARDS patients, the effects of NO inhalation on survival of patients with ARDS remain largely unknown.

Early inhaled nitric oxide therapy in premature newborns with respiratory failure

Pediatric Heart Lung Center, University of Colorado School of Medicine, and Children's Hospital, Denver

BACKGROUND
The safety and efficacy of early, low-dose, prolonged therapy with inhaled nitric oxide in premature newborns with respiratory failure are uncertain.

METHODS
We performed a multicenter, randomized trial involving 793 newborns who were 34 weeks of gestational age or less and had respiratory failure requiring mechanical ventilation. Newborns were randomly assigned to receive either inhaled nitric oxide (5 ppm) or placebo gas for 21 days or until extubation, with stratification according to birth weight (500 to 749 g, 750 to 999 g, or 1000 to 1250 g). The primary efficacy outcome was a composite of death or bronchopulmonary dysplasia at 36 weeks of postmenstrual age. Secondary safety outcomes included severe intracranial hemorrhage, periventricular leukomalacia, and ventriculomegaly.

RESULTS
Overall, there was no significant difference in the incidence of death or bronchopulmonary dysplasia between patients receiving inhaled nitric oxide and those receiving placebo (71.6 percent vs. 75.3 percent, P=0.24). However, for infants with a birth weight between 1000 and 1250 g, as compared with placebo, inhaled nitric oxide therapy reduced the incidence of bronchopulmonary dysplasia (29.8 percent vs. 59.6 percent); for the cohort overall, such treatment reduced the combined end point of intracranial hemorrhage, periventricular leukomalacia, or ventriculomegaly (17.5 percent vs. 23.9 percent, P=0.03) and of periventricular leukomalacia alone (5.2 percent vs. 9.0 percent, P=0.048). Inhaled nitric oxide therapy did not increase the incidence of pulmonary hemorrhage or other adverse events.

CONCLUSIONS
Among premature newborns with respiratory failure, low-dose inhaled nitric oxide did not reduce the overall incidence of bronchopulmonary dysplasia, except among infants with a birth weight of at least 1000 g, but it did reduce the overall risk of brain injury.

Study Shows Nitric Oxide Therapy for Newborns Effective, Economical

January 12, 2004 -- Journal Pediatrics

By Jocelyn Uhl

An inhaled treatment for critically ill newborns is less invasive, more effective, and less expensive than the treatment that has traditionally been used to treat a potentially fatal condition called hypoxic respiratory failure (HRF), according to a Pitt study published in the journal Pediatrics.

The study focuses on the positive effects of inhaled nitric oxide for the treatment of HRF and reveals a rarity in today's world of rising medical costs: a breakthrough treatment that benefits patients and is less expensive than the standard treatment.

"It's almost unprecedented to hear of an advanced medication that actually saves money compared with an older treatment," said the study's lead author, Derek C. Angus, an associate professor of critical care medicine in Pitt's School of Medicine and director of the Clinical Research, Investigation and Systems Modeling of Acute Illness (CRISMA) laboratory. "When you are treating a critically ill baby, you want the best treatment available no matter what the cost. It's heartening to learn that, in the case of babies with hypoxic respiratory failure, we can offer state-of-the-art treatment that improves outcomes in comparison to traditional care and does so at potentially reduced costs overall."

HRF develops in newborns whose lungs cannot deliver enough oxygen to their bodies, endangering their lives and causing them to appear bluish. The condition, which affects about 30,000 full-term and near-term infants each year, often appears on the first day after birth. There is no prenatal test or other way to predict which infants will develop HRF, so there is no known way to prevent the condition.

In the past, the only effective treatment for newborns with HRF who did not respond to artificial ventilation and supplemental oxygen was an invasive surgical procedure known as extracorporeal membrane oxygenation (ECMO), which involves cutting a newborn's jugular vein and putting the baby on a heart-lung machine to oxygenate the blood. Besides being invasive, the procedure has the potential to cause severe complications.

Nitric oxide, by contrast, is administered as an inhaled gas and has few potential complications. The drug works by relaxing smooth-muscle cells in blood-vessel walls in the lungs, allowing the lungs to properly oxygenate the blood and provide it to the rest of the body. But while hospital stays involving ECMO are reimbursed by private and

government insurance plans, experts say that reimbursement for inhaled nitric oxide traditionally has been inadequate. This is based in large part on the fact that it is a newer treatment than ECMO, and thus reimbursement policies have not caught up to the fact that the therapy is now more widely used.

"Many therapies and life-saving equipment readily accepted by society are quite costly," said Maria Hardin, vice president of patient services for the National Organization for Rare Disorders. "Perhaps now that we have hard data on the cost savings this treatment provides, insurers will do a better job of covering it."

The key findings of the study follow.

- For every 100 newborns with HRF, treatment with inhaled nitric oxide resulted in cost savings of more than $440,000. These savings occurred among newborns who did not need to be transferred to another hospital for ECMO treatment.
- Much of the cost savings stemmed from the avoidance of ECMO.
- Treating newborns with inhaled nitric oxide at local hospitals (rather than higher-level hospitals that also provide ECMO) was most cost effective, because when the treatment prevented the need for ECMO, it also avoided the cost of transferring the baby to the ECMO center.

Using the data from two randomized controlled trials and other real-life experiences with ECMO and inhaled nitric oxide, researchers in the CRISMA laboratory developed a cost-effectiveness model that estimated treatment outcomes and costs associated with treatment and recovery. The researchers looked at two scenarios: a base case in which babies were transferred to advanced-care hospitals where ECMO was available, and a reference case, in which nitric oxide therapy was administered at local i hospitals. Both scenarios suggested that nitric oxide therapy was cheaper and more effective than ECMO. The base case study showed savings of $1,880 per case, while the reference case showed even higher savings of $4,400 per case.

"We now have strong evidence that I think will surprise many physicians and hospitals," Angus said. "Hopefully, this will encourage everyone to take a new look at just how important this therapy is."

Nitric Oxide Therapy in Preemies

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that develops most commonly in preemies within the first four weeks after birth. In BPD cases, lungs do not work properly and babies need extra oxygen; additionally, they may also need help from a breathing machine.

Although the efficacy of nitric oxide treatment for newborns has been proven [1], the safety and usefulness of such treatment in premature babies (preemies) with respiratory failure is unclear. A recent article in the New England Journal of Medicine reported a study that detailed the effects of extended therapy with inhaled nitric oxide in premature babies. The researchers reported that in babies with a birth weight between 1000g and 1250g, the incidence of BPD decreased with nitric oxide treatment [2].

793 preemies were selected that had been born within the last 48 hours, had a gestational age of 34 weeks or less, had respiratory failure that required mechanical ventilation and had a birth weight of 500g to 1250g. The newborns were divided into groups according to their weight and randomly assigned to receive a placebo or nitric oxide for 21 days or until extubation. The usefulness of treatment was measured by death or occurrence of BPD at 36 weeks of postmenstrual age.

Overall there was no significant difference in the incidence of death or BPD between newborns receiving placebo and those receiving nitric oxide. However, in babies with birth weights between 1000g and 1250g, nitric oxide reduced the incidence of BPD (29.8% vs. 59.6%). Moreover, for the entire group, the occurrence of intracranial hemorrhage, periventricular leukomalacia or ventriculomegaly (i.e. brain injury) was reduced upon treatment as well (9.0% vs. 17.5%).

Presently the survival of preemies is estimated to be between 1-50%[4]. This new treatment has the potential to increase the survival rate of preemies. The study also indicated that brain injury was seen to have decreased in the preemies treated with nitric oxide. This may mean that future preemies will not only survive, but will also have normal development.

1. Roberts JD, Polaner DM, Lang P, Zapol WM. Inhaled nitric oxide in persistent pulmonary hypertension of the newborn. Lancet 1992;340:818-9

2. Kinsella, John P., Cutter, Gary R., Walsh, William F., Gerstmann, Dale R., Bose, Carl L., Hart, Claudia, Sekar, Kris C., Auten, Richard L., Bhutani, Vinod K., Gerdes, Jeffrey S., George, Thomas N., Southgate, W. Michael, Carriedo, Heather, Couser, Robert J., Mammel, Mark C., Hall, David C., Pappagallo, Mariann, Sardesai, Smeeta, Strain, John D., Baier, Monika, Abman, Steven H. Early Inhaled Nitric Oxide Therapy in Premature Newborns with Respiratory Failure. N Engl J Med 2006 355: 354-364

3. What is bronchopulomonary dysplasia? Online http://www.nhlbi.nih.gov/health/dci/Diseases/Bpd/Bpd_WhatIs.html. 14 August 2006.

4. http://www.tttsfoundation.org/nicuquest.html. 14 August 2006.

Toxicity

One of the primary concerns regarding the use of nitric oxide in a therapeutic modality is its potential *toxicity*. Three major areas of concern regarding toxicity have to do with: nitrogen dioxide (NO2) production, methemoglobinemia, and the production of peroxynitrite. The following discussion elaborates these types of toxicity, and explains how some concerns can be met.

Production of Nitrogen Dioxide (NO_2)

Nitric oxide itself can cause toxicity via its role in the formation of the nitrite, nitrogen dioxide (NO_2), and methemoglobin. NO_2 is produced spontaneously from NO and O_2 and is a strong oxidizer that causes lipid peroxidation in cells.

The amount of NO_2 produced depends on the amount of NO and the amount of surrounding O_2, the residence time of NO with O_2 which can be accelerated by increased NO concentrations and high FIO_2, and the square of NO concentration. The higher the FIO_2, the greater the amount of oxidation of NO to NO_2. Similarly, the higher the concentration of NO, the shorter the time to achieve oxidation to NO2. Because O_2 concentration is typically much greater than NO concentration, it is assumed that O_2 remains constant, and it is assumed that all of the conversion of NO is to NO_2.

The lethal effect of NO_2 is caused by pulmonary edema, and short-term exposure to more than 150 ppm of NO_2 is usually fatal. In the usual doses of NO, such as 0.5% to 4%, methemoglobinemia has not usually been a problem, but this exposure should be monitored closely. Although the Occupational Safety and Health Administration (OSHA) has set safety limits for NO_2 exposure at 5 ppm, parenchymal lung injury and airway reactivity have occurred with inhalation of as little as 2 ppm. As a result, the goal in clinical trials is to maintain inhaled NO_2 levels as low as possible, especially since its effects on patients with compromised or injured lungs, as in ARDS patients, are generally unknown.

Discovering safe dosage parameters for inhaled NO_2 has been the focus of numerous animal studies evaluating the parenchymal effects of high levels of inhaled NO_2 (>10 ppm). Those studies have reported the occurrence of pulmonary edema, hemorrhage, changes in surface tension properties of surfactant, reduction in number of alveoli, and even death. Even at concentrations as low as 2 ppm, inhaled NO_2 produced alveolar cell hyperplasia, altered surfactant hysteresis, changes in the epithelium of the terminal bronchiole, and loss of cilia.

Human studies investigating safe dosage parameters for inhaled NO_2 have come up with conflicting results, particularly in regard to evaluating airway responses to low dosage levels. Some have even reported airway responsiveness increases at inhaled NO_2 2 ppm. Since it reacts with water to produce nitric acid and undergoes irreversible absorption by the pulmonary epithelial lining fluid, inhaled NO_2 may remain in the lungs for prolonged periods of time. Therefore, measuring exhaled NO_2 may not provide a sensitive indicator of toxic pulmonary levels.

In clinical trials utilizing mechanical ventilation systems, researchers have found that the NO_2 concentration is greater with increased NO concentration, higher FIO_2, or lower VE. They have also found that the type of ventilator also affects the amount the NO_2 concentration delivered to the patient. Because of increased residence time of NO with O_2, ventilators with a higher internal volume, like the Servo 900C, deliver a higher concentration than those with a lower internal volume, like the Puritan-Bennett 7200ae.

Clinical researchers are looking into problems associated with the potential for NO conversion to NO_2 in lung units with long residence times. Researchers have found that when inhaled NO is administered to patients with ARDS, levels of exhaled nitric oxide of 50-75% of inhaled levels are commonplace. They also note that at the NO concentration commonly used in treating ARDS (<20 ppm), a relatively long residence time (4.6-6.8 minutes) is required to produce 2 ppm NO_2. ARDS treatments rarely include such extended residence times, however, they might occur in patients with severe chronic airflow obstruction. Additionally, it remains uncertain as to whether conversion of NO to NO_2 does occur in the lungs during NO administration.

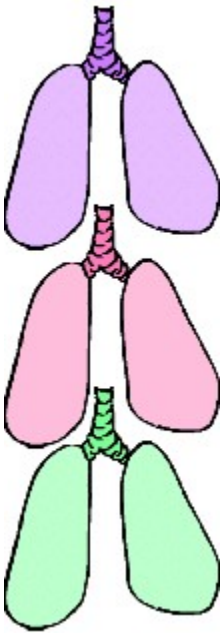
In an attempt to counter the potential problem of NO_2 in ventilators, some clinical researchers have tried using a soda lime absorber in the inspiratory circuit can be used to remove NO_2. They found that a 3 ppm NO_2 decreased to less than 1 ppm after passing through the absorber, however, they note that since soda lime also absorbs NO, its effectiveness decreases over time, and some forms of soda lime may be ineffective in removal of NO_2. In addition, they found that using a soda

lime canister during mechanical ventilation can have adverse results, such as increasing:

- the risk of system leaks
- compression volume
- resistance to breathing

They also found that using the soda lime canister with mechanical ventilation made triggering of the ventilator more difficult, and caused modification of the patient's inspiratory flow waveforms.

Methemoglobinemia



Methemoglobin is a compound formed from hemoglobin by oxidation of the ferrous to the ferric state with essentially ionic bonds. A small amount of methemoglobin is present in the blood normally, but injury or toxic agents convert a larger proportion of hemoglobin into methemoglobin, which does not function reversibly as an oxygen carrier.

A number of oxidizing agents can produce methemoglobinemia. A common cause of methemoglobinemia is exposure to nitrates. Methemoglobin reductase within the erythrocyte converts endogenously produced methemoglobin to normal hemoglobin. NADH-methemoglobin reductase is predominant and reduces two thirds of the metHb produced.

Unlike any other gas, NO binds to Fe^{++} and Fe^{+++}. When the iron in heme is oxidized from Fe^{++} to Fe^{+++}, methemoglobin is produced. Iron, in the oxidized form, cannot bind to O_2 and the affinity of the other heme groups for O_2 increases. Normal metHb levels may be due, in part, to metabolism of endogenous NO.

Production of methemoglobin by NO exposure and the high affinity of hemoglobin for NO explains the selectivity for inhaled NO on the pulmonary vasculature. Animal studies have shown that high concentrations of inhaled NO (>2%) rapidly produce methemoglobinemia, and even death in many cases. However, some animal studies have shown that methemoglobinemia does not constitute a problem at the lower doses commonly used with ARDS (<20 ppm), and similar results have been reported in humans. Clinical researchers recommend monitoring methemoglobin levels daily for patients receiving inhaled NO. If a rare upward trend in the methemoglobin level occurs,

they indicate that this can usually be controlled by using a lower, but still effective NO dose.

Measurement of methemoglobin levels is facilitated by the fact that the light absorption of methemoglobin differs from that of normal hemoglobin. It can be measured using multiple wavelength spectrophotometer (CO-oximetry) which uses only two wave-lengths and cannot distinguish between oxyhemoglobin and methemoglobin, its accuracy is affected by the presence of methemoglobin. When the reducing agent methylene blue is infused for treatment of methemoglobinemia, the accuracy of pulse oximetry may be effected due to the effects of circulating methylene blue on light absorption at the wavelengths used by pulse oximeters.

Treatment of methemoglobinemia normally involves the infusion of methylene blue, which increases NADH-methemoglobin reductase, or it can be treated by administration treated with ascorbic acid (vitamin Q). Concerns that methylene blue may inhibit the effect of guanylate cyclase, and thereby counteract the effects of inhaled NO have been dissipated by animal studies showing that methylene blue did not inhibit the action of inhaled NO on guanylate cyclase.

Production of Peroxynitrite

While researchers have yet to discover much about the potential intracellular toxicity of inhaled NO at the doses used with ARDS, it is known that superoxide (O_2-) reacts with NO to produce peroxynitrite (ONOO-), a strong oxidant which readily catalyzes membrane lipid peroxidation. However, some studies speculate it is possible that inhaled NO may also scavenge O_2- by this pathway, thus decreasing oxidant-induced lung injury. Therefore, more studies are needed to determine

whether peroxynitrite production is helpful or harmful to patients with acute lung injury.

Effects of Nitric Oxide

Nitric oxide has broad sweeping effects on the human body, and the following discussion presents you with an overview of research findings regarding those effects.

Effect on the Pulmonary Circulation
In patients with normal pulmonary hemodynamics and vascular resistance, inhaling NO has no effect on their pulmonary artery pressure or gas exchange. However, researchers have found that breathing 12% oxygen causes hypoxic pulmonary vasoconstriction caused and increases mean pulmonary artery pressure (PAP) from 14.7 + 0.8 to 19.8 + 0.9 mm Hg in healthy adults. They found that by adding 40 ppm of NO to the gas mixture, this effect could be reversed, and that because NO was inactivated locally by hemoglobin, there was no change in systemic vascular resistance.

Selective Pulmonary Vasodilation
In pharmacology, the term selective means that a drug or agent has a high degree of *selectivity*. Selectivity means that the dose of a drug produces the desired effect in the desired area of the body, without adverse effects. When the term *selective vasodilation* is used to describe a drug's action, it generally indicates two physiologic phenomena:

1. Selective pulmonary vasodilators reduce pulmonary vascular resistance without affecting systemic vascular resistance.
2. Only vascular resistance near ventilated alveoli are affected by selective pulmonary vasodilators, and inspired vasodilators are delivered to those lung units that have been ventilated. While nitric oxide itself in not a selective pulmonary vasodilator, but it becomes one when inhaled.

Studies of both animals and humans have shown that inhaled NO selectively improves blood flow to ventilated alveoli. The subjects in the studies showed reductions in intrapulmonary shunt, and improved arterial oxygenation. In subjects with ARDS, NO inhalation resulted in improved V/Q distribution measured by multiple inert-gas-elimination technique (MIGET).

Similar studies of subjects with pulmonary hypertension showed that inhaled NO decreases pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR) without affecting systemic blood pressure or

systemic vascular resistance (SVR). Results also showed that inhaled NO can reverse hypoxic-pulmonary hypertension without systemic effects.

Inhaled NO's selectivity for pulmonary vasodilation is mainly due hemoglobin's high affinity for NO, which is about 1 x 10^6 greater than the affinity of hemoglobin for O_2. As discussed earlier, NO binds to hemoglobin to form nitrosylhemoglobin, which is rapidly oxidized to methemoglobin (metHb), yielding residual nitrates and nitrites. The metHb is then converted to reduced hemoglobin due to the action of metHb reductase. As a result, methemoglobinemia is (as discussed earlier) a potential complication of inhaled NO therapy.

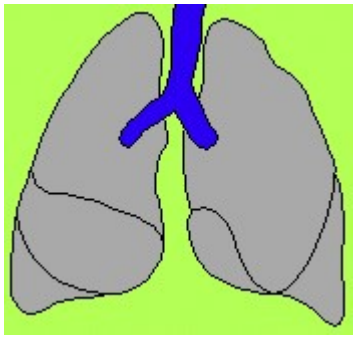
Intravenous vasodilators like sodium nitroprusside, nitroglycerin, prostacyclin are not at all selective like NO, because while they lower PAP, they also lower SVR and blood pressure. They also cause increased blood flow to both ventilated and unventilated lung units, which in turn causes increased intrapulmonary shunt and a lower partial pressure for oxygen in the arterial blood (PaO2). As you recall, one of the qualities of *selectivity* is that in pulmonary vasodilators is that they reduce pulmonary vascular resistance without affecting systemic vascular resistance.

Adverse Effects of Inhaled NO

Not all of inhaled NO's effects are positive, and the following discussion identifies some of its negative effects:

Platelet Inhibition

Numerous studies have demonstrated NO's capacity for inhibition of platelet aggregation. Researchers report that NO causes anti-platelet effects by activating guanylate cyclase, thereby increasing platelet cGMP, resulting in the inhibition of platelet adhesion, aggregation, and agglutination.



Studies of inhaled NO's effect on platelet aggregation in patients with ARDS showed significant decreases in platelet aggregation and agglutination that was not dose-dependent, with maximal inhibition occurring at 3 ppm. However, the results showed that the anti-thrombotic effect was not associated with any changes in bleeding time, hence reducing the potential clinical importance of this effect on ARDS patients.

Increased Left Ventricular Filling Pressure

Studies have shown that high doses (40-80 ppm) of inhaled NO administered to patients with left ventricular dysfunction decreases PVR and elevates pulmonary capillary wedge pressure. However, since that reduction in right ventricular afterload likely causes increased right ventricular output, resulting in resultant acute increase in left ventricular filling which could produce pulmonary edema in patients with pre-existing left ventricular dysfunction. Therefore, the studied concluded that the use of inhaled NO, even at lower doses, should be avoided in patients with documented left ventricular failure.

Rebound Hypoxemia & Pulmonary Hypertension

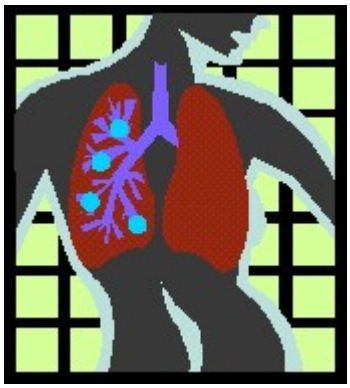
Studies have shown that withdrawal of inhaled NO could present a problem in some patients, in that there is a decrease in arterial oxygenation and an increase in PAP with daily trials of NO withdrawal. Other researchers found that rebound pulmonary hypertension commonly occurred with withdrawal of inhaled NO.

Specifically, they report finding that a transient (4-8 h)deterioration in oxygenation occurs with withdrawal of inhaled NO, and that potentially life-threatening pulmonary hypertension develops in some patients. While the reasons for this rebound effect are not entirely known, the researchers speculate that it may relate to feedback inhibition of NO synthase activity.

In order to avoid the deleterious effects of rebound during withdrawal of inhaled NO, the researchers recommend:

- Decreasing the NO to the lowest effective dose (5ppm).
- Withdrawing inhaled NO only after the patient's clinical status has improved sufficiently (e.g., FIO2 = 0.40, PEEP = 5 cm H2O, hemodynamically stable).
- Increasing the FIO2 to 0.60-0.70 before withdrawal of inhaled NO, and preparing to support the patient's hemodynamics if necessary.

Indications for Nitric Oxide Therapy



Although nitric oxide had an orphan drug designation when this CEU course was first published, this current version upates that information. NO is being used experimentally in a variety of clinical situations, including certain forms of pulmonary hypertension. When very low concentrations (<40 ppm) of NO are mixed with oxygen, the mixture selectively dilates the pulmonary blood vessels, which has proved beneficial in treating persistent pulmonary hypertension of the newborn (PPHN) and the Adult Respiratory Distress Syndrome (ARDS).

Regarding treating newborns, inhaled NO therapy is being used in clinical trials at centers where ECMO is readily available. However, there are rather stringent restrictions on the conditions under which NO therapy may be administered. In addition to having PPHN as documented by echocardiogram, a patient must also be greater than 35 weeks gestation, weigh at least 2000 gm at birth, and have previously failed to respond to maximum conventional therapies.

There is currently a great deal of enthusiasm regarding the potential benefits for using inhaled NO in the treatment of ARDS. The current treatment for ARDS is largely supportive, and has had little impact on mortality rates over the past 20 years. Since ARDS is characterized by intrapulmonary shunting, increased dead space ventilation, and pulmonary hypertension, the current therapeutic protocol consists of supplemental O_2, PEEP, and high mean airway pressure. The problem with that approach to ARDS treatment is that the administration of O2 and high airway pressure may itself worsen the high-permeability, noncardiogenic pulmonary edema associated with ARDS.

The optimism associated with inhaled NO therapy is that its administration should produce selective pulmonary vasodilation that results in improved distribution of ventilation, less shunting, improved arterial oxygenation, and lower PVR. While many of the clinical reports being published are encouraging, inhaled NO's role in the outcome of ARDS remains unclear pending the completion of the prospective, randomized, double-blind trials currently being conducted at large multi-center health care facilities.

The Table below illustrates some of the clinical situations for which the use of inhaled NO therapy is being contemplated and studied:

Table 3. Potential Clinical Uses for Inhaled Nitric Oxide

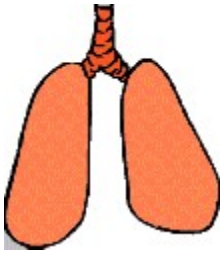
ARDS

Persistent pulmonary hypertension of the newborn
Primary pulmonary hypertension
Pulmonary hypertension following cardiac surgery
Cardiac transplantation
Lung transplantation
Acute pulmonary embolism
COPD and chronic pulmonary fibrosis
Bronchodilation
Congenital diaphragmatic hernia
Congenital heart disease

As you can see, there are a wide variety of potential opportunities for beneficial therapeutic use of inhaled nitric oxide. However, nearly all of the research studies reviewed in this CEU conclude that more research is needed before this potentially hazardous gas becomes accepted for general therapeutic application.

Administration of Inhaled NO Therapy: Safety Recommendations

While studies have shown that nitric oxide can be administered as a gas for inhalation in concentrations up to 80 ppm, the recommended dosage is <20 ppm or less. As discussed earlier, inhaled NO is delivered to ventilated lung units and does not increase V/Q mismatch, and since it is rapidly and locally inactivated by hemoglobin, there is no systemic vasodilation or hypotension.



While inhaled NO is not yet approved for regular therapeutic use, the experimental studies have reached a variety of preliminary conclusions regarding how it should be most appropriately administered. Some of the guidelines recommended regarding the safe administration of inhaled nitric oxide therapy include:

- Commercially available stock tanks not exceeding 1000 ppm of NO in nitrogen should be used.
- The blending and delivery systems need to be designed and tested for accurate NO delivery, with a minimum of NO_2 production.
- Inhaled NO and NO_2 should be continuously monitored using chemoluminescence or electrochemical analyzers. Blood methemoglobin levels should be measured frequently.
- The minimum effective concentration of NO should be used.
- In order to prevent arterial desaturation and pulmonary hypertension, weaning from NO should be gradual.

The following guidelines have been established by the American Academy of Pediatrics:

GUIDELINE OBJECTIVE(S)
To address the conditions under which inhaled nitric oxide should be administered to the neonate with hypoxic respiratory failure.

TARGET POPULATION
Term and near-term newborns with hypoxic respiratory failure.

INTERVENTIONS AND PRACTICES CONSIDERED
Inhaled nitric oxide

MAJOR OUTCOMES CONSIDERED
- Oxygenation
- Need for extracorporeal membrane oxygenation therapy
- Chronic lung disease

MAJOR RECOMMENDATIONS
1. Infants with progressive hypoxic respiratory failure should be cared for in centers with the expertise and experience to provide multiple modes of ventilatory support and rescue therapies or be transferred in a timely manner to such an institution.
2. Inhaled nitric oxide therapy should be given using the indications, dosing, administration, and monitoring guidelines outlined on the product label (further information is available from the U.S. Food and Drug Administration (FDA) Web site). An echocardiogram to rule out congenital heart disease is recommended. Center-specific criteria for treatment failure should be developed to facilitate timely consideration of alternative therapies.
3. Inhaled nitric oxide therapy should be directed by physicians qualified by education and experience in its use and offered only at

centers that are qualified to provide multisystem support, generally including on-site extracorporeal membrane oxygenation capability.

4. Generally, inhaled nitric oxide should be initiated in centers with extracorporeal membrane oxygenation capability. If inhaled nitric oxide is offered by a center without extracorporeal membrane oxygenation capability, for geographic or other compelling reasons, mutually acceptable treatment failure criteria and mechanisms for timely transfer of infants to a collaborating extracorporeal membrane oxygenation center should be established prospectively. Transfer must be accomplished without interruption of inhaled nitric oxide therapy.
5. Centers that provide inhaled nitric oxide therapy should provide comprehensive long-term medical and neurodevelopmental follow-up.
6. Centers that provide inhaled nitric oxide therapy should establish prospective data collection for treatment time course, toxic effects, treatment failure, use of alternative therapies, and outcomes.
7. Administration of inhaled nitric oxide for indications other than those approved by FDA or in other neonatal populations, including compassionate use, remains experimental. As such, inhaled nitric oxide should be administered according to a formal protocol that has been approved by FDA and the institutional review board and with informed parental consent.

POTENTIAL BENEFITS

- Improved oxygenation.
- Reduced need for extracorporeal membrane oxygenation without increased neurodevelopmental, behavioral, or medical abnormalities at 2 years of age.
- Reduced incidence of chronic lung disease.

POTENTIAL HARMS

Potential toxic effects*:* Methemoglobinemia (secondary to excess nitric oxide concentrations), direct pulmonary injury (attributable to excess levels of nitrogen dioxide), and ambient air contamination.

QUALIFYING STATEMENTS

The recommendations in this statement do not indicate an exclusive course of treatment or serve as a standard of medical care. Variations, taking into account individual circumstances, may be appropriate.

Because hypoxic respiratory failure is often rapidly progressive and abrupt, discontinuation of inhaled nitric oxide may lead to worsening oxygenation, the risk of delayed provision of extracorporeal membrane oxygenation must be considered carefully when determining the appropriate time of transfer.

Additional large randomized trials of inhaled nitric oxide in premature neonates are required because they may experience more toxic effects than term and near-term infants.

As discussed earlier, that last recommendation is particularly important when inhaled NO is being used to manage pulmonary hypertension because of the potential for rebound hypertension, which can be severe and cause oxygen desaturation.

Researchers speculate that the rebound hypertension may be caused by a down-regulating effect endogenous NO production in the pulmonary endothelium. Since the vasodilating effect of inhaled NO ends with the removal of the gas, an increase in FIO_2 up to 1.0 may be needed as the inhaled NO is terminated. Subsequently, the FIO_2 can be reduced over the next few hours as pulmonary hemodynamics restabilize, and close monitoring of arterial oxygenation is critical when weaning from NO.

NO is usually available in H-cylinders that contain 450-1000 parts per million (ppm) of NO mixed with nitrogen. Once NO comes in contact with high concentrations of oxygen, it forms nitrogen dioxide (NO_2) which is very irritating to lung tissue. Most studies recommend minimizing the production of NO_2 by introducing NO to the inspiratory limb of the mechanical ventilator just proximal to the ETT. This reduces the contact time between NO and O_2, and in the patient's bloodstream, excess NO is quickly absorbed by hemoglobin, forming methemoglobin.

Delivery Systems

While there are limited commercial systems currently available for delivery of inhaled NO, the various health care centers participating in the clinical trials have built their own systems. Those systems tend to vary considerably, but some of the more important considerations they all have had to deal with include:

- **Dependability and Safety:** Patient safety is a major issue because inhaled NO is used with the most critically ill, and the complex delivery systems are vulnerable to errors that could compromise ventilation, oxygenation, or delivery of the correct NO concentration. Prior to patient use, the NO delivery systems need to be thoroughly evaluated in the laboratory to ensure they are functioning properly.

- **Precision and Stability of Dosage Delivery:** In order to avoid the hazardous complications that this CEU has already described as being associated with inhaled NO therapy, it is vital to ensure that the desired dose is delivered precisely and on a stable basis. In the clinical studies currently being conducted, NO is usually supplied from a cylinder with a high NO concentration (800-2200 ppm of NO in N_2). Prior to delivery to the patient, it is diluted with N_2, air, or O_2. Safety and convenience are generally the deciding factors when selecting the cylinder's concentration. Cylinders with higher concentrations are convenient because they mean fewer cylinder changes and/or smaller cylinders, but they also increase the risk of exposure to high NO and NO_2 for both the patient and care givers. Cylinders with a higher NO concentration also allow delivery of a higher FIO_2 due to less dilution from the NO source.

- **Minimize Production of NO2:** All of the clinical studies emphasize the importance of maintaining inhaled NO_2 as low as possible (2 ppm). The risk of significant NO_2 production at NO doses 20 ppm in adult ventilator systems is not significant unless the FIO_2 is high (>0.9) and the minute ventilation is low (<5 L/min). As mentioned earlier, some clinical studies recommend the use of soda lime to limit NO_2 delivery, however, a properly designed system can minimize significant NO_2 generation.
- **Monitoring NO and NO_2:** While many clinical researchers using inhaled NO therapies have developed nomograms to assist with

proper dosing, the blenders used to mix NO, N_2, and O_2 are very imprecise and, to make matters more imprecise, none of which have been calibrated for use with NO. This makes it all the more important to directly monitor the delivery of inhaled NO and the potentially injurious inhaled NO_2.

- **Design for Scavenging of NO:** As a result of concerns regarding potential contamination of the environment with NO and NO_2 and for adverse effects on healthcare providers at the bedside, OSHA has established exposure limits for NO at a time-weighted average of 25 ppm for 8 hours in the workplace. This provides practitioners some leeway since that limit is higher than the typical NO dose (<20 ppm). In intensive care environments that have >6 air exchanges/h, ambient NO levels should remain very low. While many clinical trial sites report that that ambient NO levels are low (>0.25 ppm) during NO administration with or without scavenging, it is still recommended that systems be designed to permit scavenging of the exhaled gases during NO therapy.

- Even with scavenging, it is important to remember that scavenging gases from the exhalation port of the ventilator does not completely eliminate ambient contamination because the ventilator may internally leak gas containing NO as part of its normal operation. Also, it is important that the scavenging system be constructed in a way that it does not affect ventilator function or increase the expiratory resistance.

- **Maintain Ventilator Function:** Precautions need to be taken to assure that ventilator function, particularly the alarm systems, not be affected when adapting the ventilator to permit delivery of inhaled NO. Since adding NO lowers the FIO_2, O_2 concentration needs to be monitored distal to the site of NO titration into the system, recalling that it is impossible to deliver 100% O_2 during inhaled NO therapy. While some studies identified concerns that exposure to NO might have a negative effect on the internal components of the ventilator and the external blenders and flow meters, there are no published reports of damage or malfunction of equipment related to NO exposure, even after considerable exposure time during clinical trials.

- When NO is used inline with mechanical ventilation, the amount of gas introduced into the circuit is regulated by a flow controller, and concentrations of NO, NO_2 and FIO_2 are all measured inline near the ETT. The recommended protocols for therapeutic administration of inhaled NO vary among the sites performing the clinical trials, however their common goal is to balance the amount of inhaled NO and NO_2 to maximize the vasodilatory effects while minimizing methemoglobinemia and the toxic effects of NO2. The ideal

concentrations for treating adult patients are generally agreed to be amounts of 5 to 10 ppm on NO, however, there have been some reports of using concentrations up to 80 ppm with no side effects.

Techniques for NO Delivery

Adult Mechanical Ventilators

Continuous administration into the inspiratory limb of the ventilator circuit is one of the most common and simplest techniques for delivering NO. Monitoring the mean NO concentration delivered to the patient is accomplished by estimating from the NO flow and the inspiratory flow. This technique does not work well with phasic-flow adult ventilators because with such systems, the inspiratory circuit fills with NO during expiration and delivers a large bolus of NO to patients with the beginning of each breath.

When NO is continuously added at the Y-piece, the inspiratory circuit does not fill with NO during expiration, but rather bleeds out the expiratory limb of the ventilator during expiration. Also, measuring the inspired NO concentration is not possible with this method, and the dose can only be approximated by mathematical calculation. Researchers report that this system suffers many of the same limitations of NO titration into the inspiratory circuit--specifically, augmentation of tidal volume, decreased ability to trigger the ventilator, and changes in delivered concentration of NO with changes in inspiratory flow.

Some clinical researchers have tried delivering a constant flow of NO through the endotracheal tube into the trachea. However, the problems associated with this method are similar to titration at the Y-piece, and it can be dangerous because during accidental disconnection, O_2-free NO gas continues to flow into the trachea and quickly produces hypoxia.

Another technique used in clinical studies involves injecting NO into the ventilator circuit during the inspiratory phase by using a nebulizer-drive mechanism that operates during inspiration. The NO required to achieve

the desired patient dose is contained in the gas supply to the nebulizer after being mixed with gas delivered from the ventilator. This method delivers a consistent NO dose only with constant flow ventilation because the gas flow from the nebulizer is constant during inspiration, however, it does not work well with varying inspiratory flow patterns such as pressure control.

Another shortcoming of this method is that it cannot deliver a stable dose with ventilatory modes such as pressure support in which tidal volume and inspiratory time vary from breath to breath. This technique's primary advantage is that it minimizes NO_2 generation because the residence of NO in an O_2-containing gas mixture is minimized. On the other hand, precise control of the inspired NO concentration is not possible with this technique. In order for this technique to be acceptable, flow from the ventilator must be continuously and precisely measured, and the injected dose of NO must be precisely titrated so that the delivered NO and inspiratory flow waveform are not affected.

Another approach described by research studies involves administer-in inhaled NO to adult mechanically ventilated patients by premixing the NO with N_2 (or air), introducing the mixture proximal to the gas inlet of the ventilator. Typically these systems report adding the O_2-N_2-NO gas mixture to the low-flow inlet of the Servo 900C ventilator or the high-pressure air or O_2 inlet of a ventilator such as the Puritan-Bennett 7200.

One center with the Puritan-Bennett 7200 reported using an air-O_2 blender to add 800 ppm NO to the O_2 inlet of the blender, then adding N_2 or air to the air inlet of the blender. Selection of air or N_2 as the diluent is determined by the extent of NO_2 generation. NO must be mixed with N_2 with high NO doses (>20 ppm), high FIO_2 (e.g., >0.90), or low minute ventilation (<5 L/min). The gas mixture coming out of the blender is delivered to the high pressure air inlet of the ventilator, and the NO concentration delivered to the patient is determined by the FIO_2 settings on the external blender and the ventilator, confirming the delivered NO concentration by direct measurement.

Several researchers report that it is also important to analyze FIO_2 due to the effect of NO/N_2 dilution on delivered FIO_2 because air-O_2 blenders do not always deliver a precise NO concentration. This is particularly true when flow from one of two or more blenders in series is less than that required for accurate gas mixing. While multiple blenders in series can be used to reduce the NO concentration, several centers report finding such systems to be superfluous and unnecessarily complex.

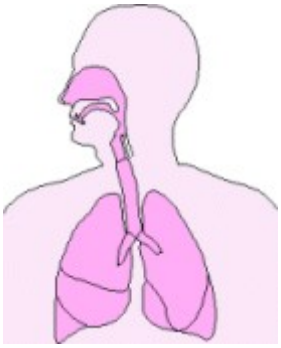
The reported advantage of premixing before introduction into the ventilator is that the NO dose is constant throughout inspiration and unaffected by changes in minute ventilation or flow waveform. A potential disadvantage of this technique is that the NO dose changes

when the ventilator FIO_2 setting is changed. However, that can be compensated for by adjusting the setting on the external blender.

Yet another potential problem with this technique arises because the gas used to power the nebulizer with some ventilators is either air or O_2, depending, on the FIO_2. The delivered NO concentration can be affected by activation of the nebulizer for bronchodilator delivery because NO is added via the air inlet of the ventilator. However, using an inhaler instead of a nebulizer can prevent this problem from arising.

Since current-generation ventilators use microprocessor technology to set the delivered oxygen concentration, researchers point out that it seems reasonable to use similar technology for the delivery of NO. They suggest that coupled with precision solenoids, microprocessor technology could be used to mix air, $_2$ and NO to achieve the desired FIO_2 and NO concentration.

Techniques for NO Delivery



Pediatric Mechanical Ventilators
NO can also be titrated into the inspiratory limb of the ventilator circuit of the continuous-flow ventilators used in pediatrics. Clinical studies indicate that titration should take place near the ventilator outlet to ensure adequate mixing before reaching the patient, and the oxygen concentrations should be analyzed distal to the point of NO introduction into the system.

As a result, generation of NO_2 is kept to a minimum since the residence time in the system is short. The following formula can be used to calculate the estimated NO concentration:

$$[NO] = \frac{(\text{NO flow})\ (\text{source ppm})}{(\text{NO flow} + \text{ventilator flow})}$$

However, because this is just an estimation, it is still necessary to measure the NO concentration actually delivered since that dose will remain constant provided that the total flow through the system does not change. Clincal sites using this approach have reported it to be an effective and reliable technique.

Manual Ventilators

In addition to use in mechanical ventilators, clinical studies indicate that for manual ventilation, NO can be diluted with O_2 and introduced into the gas inlet port of the manual ventilator. They also report using this type of system with self-inflating resuscitators as well as gas-inflating resuscitators. The following formula can be used to calculate the estimated NO concentration:

$$[NO] = \frac{(\text{N flow})\ (\text{source ppm})}{(\text{NO flow} + O_2 \text{ flow})}$$

When using this technique for ventilating the patient, assuring that the correct dose of NO concentration is being delivered is particularly important because NO delivery may change with changes in minute ventilation if the flow into the manual ventilator is less than the minute ventilation.

Clinical studies report that since this system is generally used intermittently for bedside manual ventilation and during patient transport for diagnostic procedures, care must be taken to avoid NO_2 generation between uses--particularly if the resuscitator is not used for a prolonged time. Flushing the resuscitator with O_2 before and after each use can help avoid this problem.

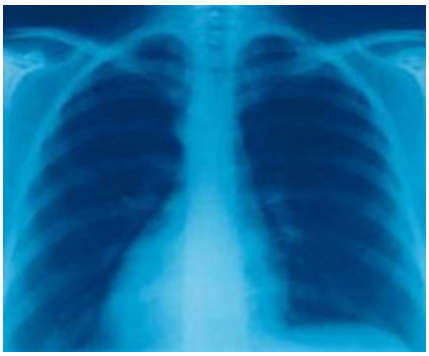
Spontaneous Breathing

Inhaled NO can be delivered to those ARDS patients who are capable of spontaneously breathing, and clinical studies report using several types of systems including one with a tight-fitting face mask. The exhaled gas in these systems can be scavenged by the hospital vacuum system, but because of the risk of NO_2 generation within the bag, reservoir bags should be avoided.

Spontaneously breathing patients using a transtracheal O_2 catheter or a nasal cannula are also candidates for inhaled NO therapy, however there are several limitations to these systems, including:

- it is not possible to analyze the delivered dose
- the dose varies with the ventilatory pattern of the patient

Dosage

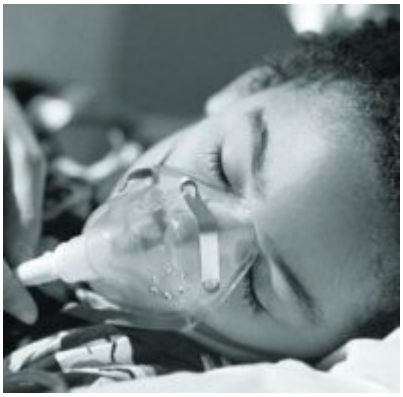


Initial clinical studies using inhaled NO therapy indicated that showed it improved oxygenation in patients with ARDS at doses at 20 ppm. Subsequently there have been a variety of reports related to ideal dosage and doses that produce 50% of the desired effect (ED50), and some conclusions reported include:

- Several studies have reported effective doses at <5 ppm.
- Another study of ARDS patients found an ED50 100 ppb for improvement of oxygenation and an ED50 of 2-3 ppm for improvement of pulmonary artery pressure--with beneficial effects peaking at about 10 ppm.
- One study found that it was most appropriate to initiate inhaled NO therapy at a dose of 20 ppm, and if oxygenation improves, decrease the inhaled NO to the lowest effective dose.
- Studies report that at the doses commonly used with ARDS (<20 ppm), inhaled NO is considered relatively free of toxicity.
- The clinical studies report no major complications related to inhaled NO, however they note that the potential for complications definitely exists.

Monitoring and Analysis

Monitoring and analyzing the delivered dose of inhaled NO and NO2 is crucial, regardless of the technique used to administer the therapy. The principal methods used to monitor NO and NO_2 are chemoluminescence and electrochemical analysis.



Chemoluminescence Analyzers

The traditional and well-established method for NO and NO_2 analysis is chemoluminescence, a technique originally used for many years in industrial and environmental applications, and more recently adapted to biomedical uses.

Electrochemical Analyzers

While the commonly used chemoluminescence analyzers are accurate and precise, they are also typically large, expensive, and cumbersome to use. Electrochemical cells are another option that has recently become available for measuring both NO and NO_2. Electrochemical analyzers are small, portable, rugged, designed specifically for medical applications, and are less expensive than chemoluminescence. They generate a potential difference proportion-al to the concentrations of NO and NO_2, from reactions of NO and NO_2 within an electrolyte.

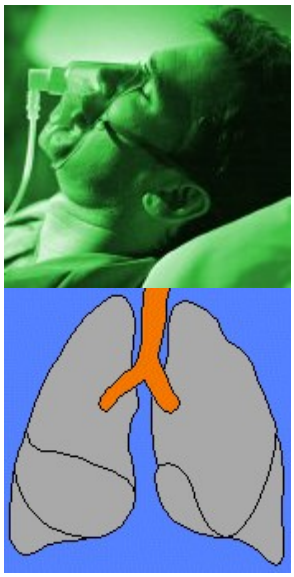
Clinical evaluations of these devices have found them to be suitably accurate for clinical use, but because electrochemical NO cells are affected by humidity, they should sample NO before the gas is humidified. These cells are also pressure sensitive, but potential problems can be minimized by using sidestream sampling techniques.

Inspired, Tracheal, & Expired Gas Analysis

Since chemoluminescence and electrochemical analyzers generally have slow response times, the displayed concentrations of NO and NO_2 are time-weighted averages and so real-time fluctuations in NO delivery are not detected by these methods. This can provide an inaccurate impression of the characteristics of the NO delivery pattern and dose. NO concentrations are measured in the inspiratory limb of the ventilator circuit because this accurately reflects the NO concentration delivered to the lungs. It is also possible to measure NO concentration in tracheal gas, but it is important to remember that tracheal NO concentration is

affected by changes in expiratory NO concentrations and by the ventilatory pattern. Whatever analysis technique is used, inhaled concentrations of NO should be measured and reported in a reproducible manner, and to enable comparisons with clinical studies NO concentration should be analyzed from the inspiratory limb of the ventilator circuit.

Summary



Nitric oxide is administered as an inhaled gas, and readily diffuses into the vascular endothelium, where it stimulates guanylyl cyclase in the cell, increases cylic GMP, and produces smooth muscle relaxation. NO also quickly diffuses into the bloodstream where it is inactivated by binding to hemoglobin, producing methemoglobin. Nitric oxide has a short half-life of less than 5 seconds since it is quickly bound by hemoglobin. In the presence of oxygen, NO is converted to nitrogen dioxide, NO_2, a nitrite toxic to the lung.

While inhaled NO therapy is not yet approved for general clinical use, respiratory care practitioners caring for critically ill patients should become familiar with this therapy. Since NO is administered by inhalation, delivery systems for NO legitimately fall within the practice of respiratory therapists. Regardless of the future role of inhaled NO as a therapeutic agent, its investigational has revealed a great deal about inhaled selective pulmonary vasodilators and the development of safe systems for their administration.

Safety of Withdrawing Inhaled Nitric Oxide Therapy **in Persistent Pulmonary Hypertension of the Newborn**

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Abstract

Objective

Because of case reports describing hypoxemia on withdrawal of inhaled nitric oxide (I-NO), we prospectively examined this safety issue in newborns with persistent pulmonary hypertension who were classified as treatment successes or failures during a course of I-NO therapy.

Methods

Randomized, placebo-controlled, double-masked, dose-response clinical trial at 25 tertiary centers from April 1994 to June 1996. Change in oxygenation and outcome (death and/or extracorporeal membrane oxygenation) during or immediately after withdrawing I-NO were the principal endpoints. Patients (n = 155) were term infants, <3 days old at study entry with echocardiographic evidence of persistent pulmonary hypertension of the newborn. Exclusion criteria included previous surfactant treatment, high-frequency ventilation, or lung hypoplasia. Withdrawal from treatment gas (0, 5, 20, or 80 ppm) started once treatment success or failure criteria were met. Withdrawal of treatment gas occurred at 20% decrements at <4 hours between steps.

Results

The patient profile was similar **for** placebo and I-NO groups. Treatment started at an oxygenation index (OI) of 25 ± 10 (mean ± SD) at 26 ± 18 hours after birth. For infants classified as treatment successes (mean duration of therapy = 88 hours, OI <10), decreases in the arterial partial pressure of oxygen (PaO_2) were observed only at the final step of withdrawal. On cessation from 1, 4, and 16 ppm, patients receiving I-NO demonstrated a dose-related reduction in PaO_2 (−11 ± 23, −28 ± 24, and −50 ± 48 mm Hg, respectively). For infants classified as treatment failures (mean duration of therapy = 10 hours), no change in OI occurred for the placebo group (−13 ± 36%, OI of 31 ± 11 after the withdrawal process); however a 42 ± 101% increase in OI to 46 ± 21 occurred for the pooled nitric oxide doses. One death was possibly related to withdrawal of I-NO.

Conclusion

For infants classified as treatment successes, a dose response between the I-NO dose and decrease in PaO_2 after discontinuing I-NO was found. A reduction in I-NO to 1 ppm before discontinuation of the drug seems to minimize the decrease in PaO_2 seen. For infants failing treatment,

discontinuation of I-NO could pose a life-threatening reduction in oxygenation should extracorporeal membrane oxygenation not be readily available or I-NO cannot be continued on transport.

There are ~10 000 term or near-term newborns in the United States per year who develop persistent pulmonary hypertension (PPHN) and/or hypoxemic respiratory failure.[1,2] Therapy for these patients has undergone a major change within the last 5 years because of the increasing use of inhaled nitric oxide (I-NO), high-frequency ventilation, and surfactant to avoid rescue with extracorporeal membrane oxygenation (ECMO). Although still considered investigational, I-NO, acting as a selective pulmonary vasodilator, has been shown to produce a sustained improvement in oxygenation[3] and reduce the need **for** ECMO.[4,5] Presently, use of I-NO therapy as an adjunct to conventional neonatal respiratory therapy is widespread.[6] NO is used at many centers without ECMO facilities, because most newborns with PPHN or hypoxemic respiratory failure are born at non-ECMO centers and develop signs of these disorders shortly after birth.[3]

In 1990, a hidden mortality associated with neonatal transports to ECMO centers was described; labile oxygenation characteristic of PPHN and late transfers to ECMO centers were implicated.[7] Now, with the increasing use of I-NO in all age groups, there have been case reports in older pediatric patients and adults describing serious and life-threatening pulmonary hypertension and hypoxemia on the withdrawal of this therapy.[8-10] We hypothesized that with evolving protocols in the treatment of PPHN, withdrawing NO could lead to serious reductions in oxygenation. This hypothesis was studied in the second part of a randomized, double-masked, placebo-controlled, dose-response, multicenter trial which evaluated the efficacy and safety of I-NO for PPHN.[3]

Methods

Enrollment occurred between April 1994 and June 1996. Fifteen of the 25 neonatal intensive care units were ECMO centers. The protocol and consent forms were approved by the local institutional review board before patient enrollment. Written informed consent was obtained for each patient before enrollment. Equipment, treatment gases, and funding based on patient recruitment at each site was provided by Ohmeda, PPD (Liberty Corner, NJ).

Hypotheses

The principal hypothesis was that withdrawal of I-NO, from newborns with PPHN who met treatment failure or success criteria, would lead to decreased oxygenation related to step-wise reductions of treatment gas dose, mandated by a weaning protocol. As a corollary hypothesis, the postulated decreases in oxygenation had clinical consequences, with regard to the need for ECMO or death.

Patient Entry Criteria
Patients were term infants (n = 155) with PPHN documented by echocardiography and were treated with an Infant Star conventional ventilator (Infrasonics, Inc, San Diego, CA). Inclusion criteria were fractional inspired oxygen concentration (FIO_2) of 1.0, mean airway pressure ≥10 cmH_2O, and a postductal arterial partial pressure of oxygen (PaO_2) of 40 to 100 mm Hg. The major exclusion criteria were previous therapy with surfactant, concomitant high-frequency ventilation, and lung hypoplasia.

Protocol for Weaning Treatment Gas Levels
All patients received treatment gas using a delivery system (Ohmeda, PPD, Madison, WI) designed expressly to deliver either NO mixed with nitrogen or nitrogen alone (placebo), into the inspiratory limb of the ventilator system using a mass flow controller. Electrochemical detectors provided a continuous measurement of NO and NO_2 (model EC90 NO monitor and model EC40 NO_2 monitor, Bedfort Scientific Ltd, Kent, England).[3]

Treatment gas consisted of either 0, 5, 20, or 80 ppm of I-NO, maintained until treatment success or failure criteria were met. The clinical investigators who managed the neonatal care were masked to treatment gas assignment through the 1-year follow-up. The unmasked laboratory investigator monitored NO, NO_2, and methemoglobin levels. The masked clinical investigator was allowed to withdraw the patient from the study if it was judged to be in the best interests of a patient who was deteriorating but had not met specific failure criteria; this accounted for 20% of patients enrolled in the study.[3] The data from this group was not included in the withdrawal analyses.

Patients were classified as a treatment failure when the PaO_2 was <40 mm Hg for 30 minutes and the FIO_2 was 0.95 on the conventional ventilator. In addition, patients were classified as treatment failures if they had refractory hypotension defined as a mean systemic arterial pressure of <35 mm Hg, independent of oxygenation. Patients were classified as a treatment success if they had a PaO_2 ≥60 mm Hg, FIO_2 <0.6, and mean airway pressure <10 cm H_2O.

Immediately after reaching treatment failure or success criteria based on a blood gas result and ventilator settings, the masked, clinical investigator ordered a 20% reduction in treatment gas. An arterial blood gas with a record of hemodynamic and ventilatory status, was obtained 15 to 30 minutes after the change. No change in mechanical ventilation was permitted. Further 20% reductions could then be made immediately, or within 4 hours. This weaning process was continued until treatment gas was turned off. At each step the same data were collected, with no ventilatory changes immediately before, and 15 to 30 minutes after, the

change. Arterial blood gases were obtained pre- and postchange during this 15- to 30-minute interval. Postchange data could be used as prechange data if a rapid weaning process was desired. If the protocol was followed, there would be 10 time points for data collection per patient during the weaning process. The treatment gas could be increased back up by 20% with appropriate increases in FIO_2 if the PaO_2 became <40 mm Hg during a weaning step. The final doses of I-NO were 20% of the starting dose (0, 5, 20, or 80 ppm), that is, 0, 1, 4, and 16 ppm.

Figure 1 depicts the different dose reductions of treatment gas which were analyzed for oxygenation during the withdrawal process.

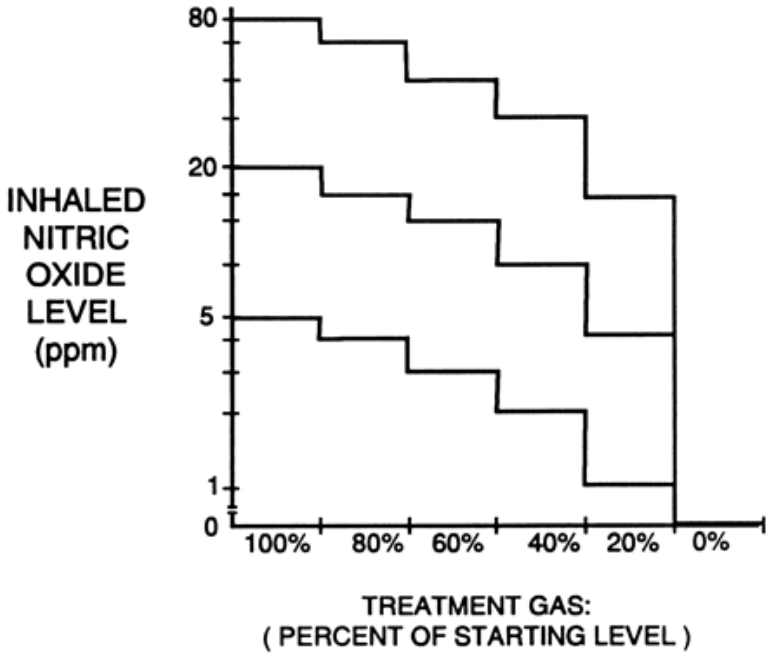

Fig. 1. Protocol for withdrawal of inhaled nitric oxide from patients with persistent pulmonary hypertension of the newborn. Patients were treated with either 0, 5, 20, or 80 ppm (masked) until treatment success or failure criteria were met. Then, 20% decrements in treatment gas were required within 4 hours if success criteria were still met. Ventilator settings were kept constant for a 15- to 30-minute period after a change in inhaled nitric oxide level. Final doses were 0, 1, 4, and 16 ppm.

Safety Monitoring

An independent data and monitoring board was composed of pediatric sub-specialists and statisticians. A safety analysis, in which the board was masked from treatment gas assignment, was performed using data from the first 100 patients.

RESULTS

Sample Size and Statistics

Changes in oxygenation on withdrawal of treatment gas to 0 ppm were studied separately **for** treatment successes and failures using the Wilcoxin rank sum test with Bonferonni correction for multiple doses of I-NO. The overall level was 0.05. Data were not used from a pre- or postchange value in oxygenation if one of the data points was missing. The Cochran-Mantel-Haenszel [2] test was used for categorical data such as death and ECMO.

Patient Profile

A detailed patient profile for this trial has been previously described.[3] The birth weight was 3.4 ± 0.5 kg (mean ± SD), 93% of the patients were outborn, and 63% had the diagnosis of meconium aspiration syndrome. Treatment gas was started at 26 ± 18 hours, when the oxygenation index (OI) ($FIO_2 \times P \times 100$/postductal PaO_2) was 25 ± 10.

Onset and Duration of Withdrawal

Table 1 demonstrates that the OI was similar for the control or the pooled NO groups, both **for** patients classified as treatment success or treatment failure. Because there were no differences in OI or duration of therapy between NO doses, pooled NO data are shown.

TABLE 1
Oxygenation Index on Reaching Success or Failure Criteria

	Control (n = 41)	Pooled I-NO (n = 114)
Treatment success	7 ± 3	6 ± 3
Treatment failure	41 ± 11	37 ± 10

Abbreviation: I-NO, inhaled nitric oxide. Oxygenation index ($FIO_2 \times$ Paw $\times$ 100/postductal PaO_2), mean ± SD.

The duration of treatment gas before reaching either treatment success or failure criteria is shown in Table 2. The average time to reach treatment failure criteria was 10 ± 13 hours for the I-NO group and this was not different from the placebo group. Patients classified as a treatment success took 81 ± 59 hours to reach weaning criteria in the I-NO group; and although it seemed to take longer to reach weaning criteria in the placebo group, this was not statistically significant (P = .13).

TABLE 2
Duration of Treatment Gas Exposure

	Control (n = 41)	Pooled I-NO (n = 114)

Failure patients		
n	13 (32%)	29 (25%)
Start treatment to withdrawal criteria (h)	10 ± 11	10 ± 13
Withdrawal process (h)	3 ± 2	11 ± 30
Success patients		
n	28 (68%)	85 (75%)
Start treatment to withdrawal criteria (h)	107 ± 69	81 ± 59
Withdrawal process (h)	11 ± 9	13 ± 14

Mean ± SD.

Acute Changes in Oxygenation

Assessments of oxygenation were obtained on an average of 9.8 of the weaning steps (5 steps with assessments before, and 15 to 30 minutes after a reduction) in patients classified as treatment successes. No changes in oxygenation were observed for reductions in treatment gas made in a step-wise manner down to 20% of the starting treatment level. However, on stopping the treatment gas, a dose-related reduction in PaO_2 was observed (Fig 2). The baseline PaO_2 (at 20% treatment gas) was not different between groups: 105 ± 39, 92 ± 26, 115 ± 41, and 129 ± 75 mm Hg (mean ± SE) for the 0-, 1-, 4-, and 16-ppm groups, respectively. On cessation of the treatment gas no change in PaO_2 for the control gas was observed. When inhaled NO at 1 ppm was stopped, there was a trend toward decreased PaO_2 (11 ± 23 mm Hg, uncorrected $P = .04$, statistically not significant). Statistically significant decreases in oxygenation, 28 ± 24 mm Hg for the 4-ppm dose and 50 ± 48 mm Hg for the 16-ppm dose, occurred. The decrease in PaO_2 was statistically greater for the 16-ppm vs. 1-ppm group.

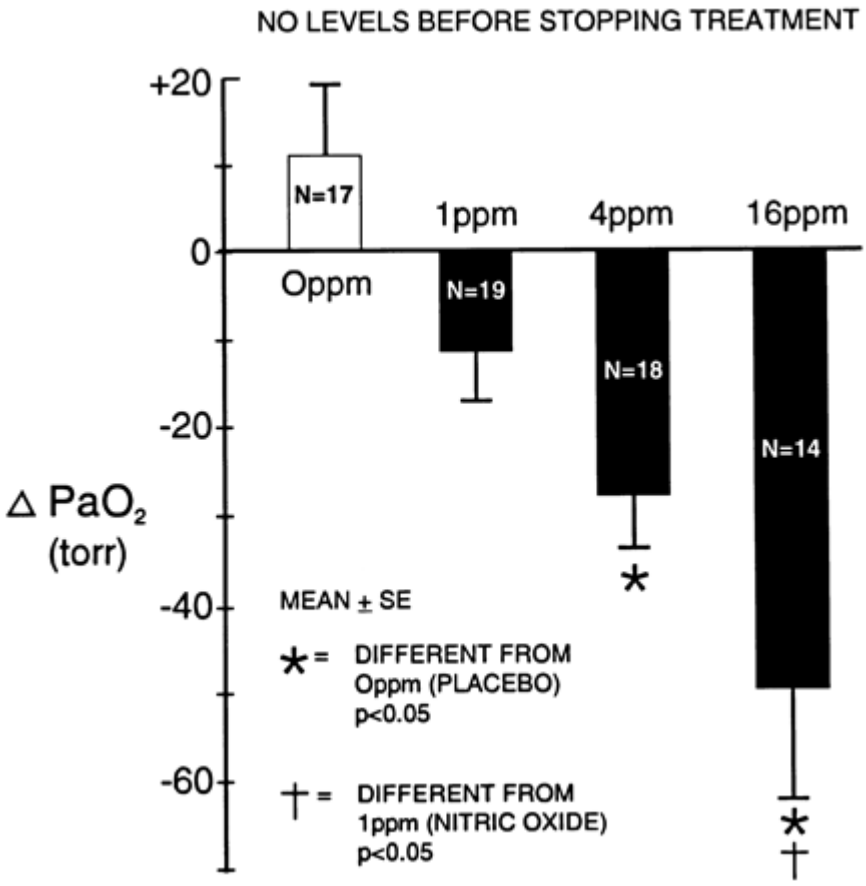

Fig. 2. Changes in PaO_2 (no ventilator changes), 30 minutes after cessation of inhaled nitric oxide treatment gas (masked) in patients with persistent pulmonary hypertension of the newborn treated successfully. Treatment success criteria were defined as FIO_2 <0.60, mean airway pressure <10 cmH_2O, and postductal PaO_2 60 mm Hg.

Because patients classified as treatment failures were acutely deteriorating, many infants had rapid weaning of the NO concentration and only 3.1 oxygenation assessments were recorded for these infants. Therefore, data were available from the start of the weaning process to cessation of treatment gas as shown in Fig 3. Because ventilator changes were made from start to finish of the weaning process, the OI was used to examine the effect of NO withdrawal. There was no significant change from baseline OI index for the placebo group after the weaning process, in patients classified as treatment failures. For the pooled NO group there was a 42 ± 101% (Fig 3) increase in OI to 46 ± 21, and this was significantly different from the placebo group (P = .03).

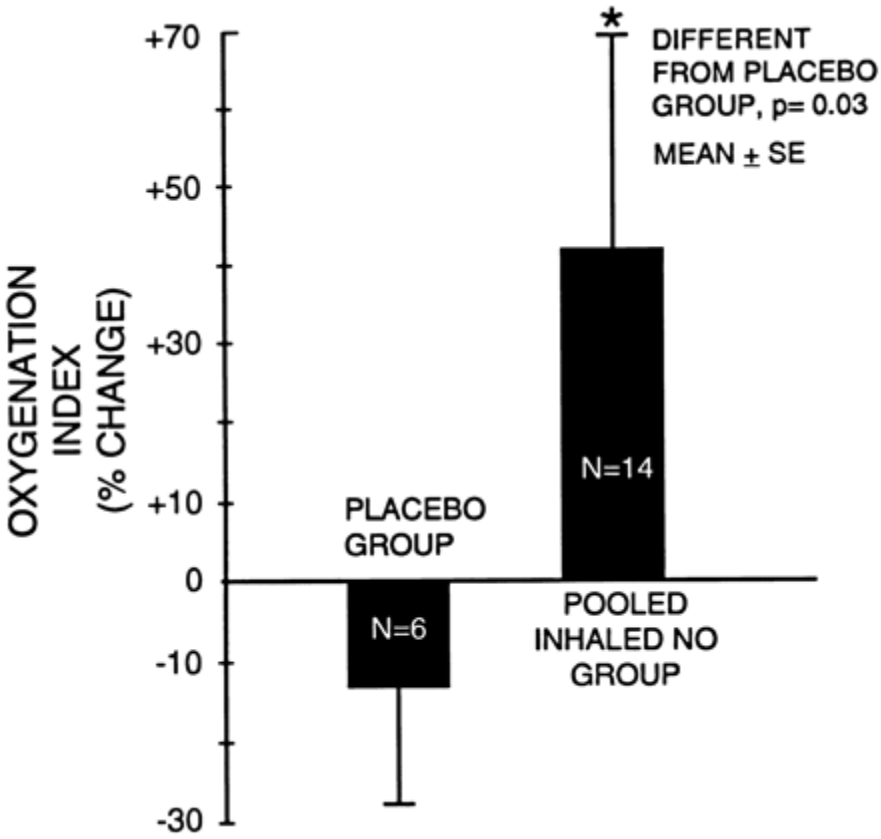

Fig. 3. Percent change in oxygenation on withdrawal of placebo or nitric oxide study gas (5, 20, or 80 ppm) in persistent pulmonary hypertension patients declared treatment failures. Patients met treatment failure criteria when the PaO_2 was <40 mm Hg for 30 minutes and/or mean systemic arterial pressure was <35 mm Hg. At the final step in the masked withdrawal protocol, patients were at 0, 1, 4, or 16 ppm. Oxygenation was assessed at the start and 30 minutes after the end of the withdrawal protocol.

The incidence of death or need for ECMO after withdrawal from NO was examined for treatment failures. There were no statistically significant differences in the placebo and pooled NO group for death or ECMO (Table 3). Of the 4 deaths in the I-NO group, 3 patients expired 7 to 21 days after the cessation of NO and investigators believed there was no relation to the treatment gas.[3] However, 1 treatment failure patient in the 20-ppm NO group was started on high-frequency oscillation rescue therapy after I-NO was discontinued. This patient expired because, in part, of a pneumothorax 11/2 hours after NO was stopped. The masked investigators determined that the death was possibly related to the treatment gas.

TABLE 3

Outcomes of Treatment Failures

	Control (n = 13)	Pooled NO (n = 29)
Death	0%	14%*
ECMO	69%	48%

Abbreviations: NO, nitric oxide; ECMO, extracorporeal membrane oxygenation.

* 3 patients, 7 to 21 days after stopping treatment; 1 patient, 1.5 hours after stopping treatment. No statistical differences, control versus pooled NO.

DISCUSSION

This prospective, randomized, placebo-controlled, double-masked, dose-response trial, is unique among studies of I-NO therapy. The study results are specific to withdrawal of I-NO from patients with PPHN on intermittent mandatory ventilation and not confounded by surfactant and high-frequency oscillatory ventilation.[11] The patients in the present study were full-term newborns started on treatment gas at a mean age of 26 hours with a mean OI of 25.[3] Their treatment was started early while they were seriously ill but not meeting ECMO oxygenation criteria. Data related to withdrawing treatment gas were analyzed for two prospectively-defined groups, infants experiencing treatment success and those with treatment failure. Accordingly, the results of this study should be useful in the development of practice guidelines for treating PPHN with I-NO as an adjunct to conventional respiratory therapy.

Patients classified as treatment successes were weaned off treatment gas generally after 3 to 4 days and investigators took 12 hours on average, to make the five step-wise, 20% reductions in treatment gas. As designed, the weaning process began when respiratory status appreciably improved (OI <10). There was excellent compliance to weaning guidelines for treatment successes. No significant changes in oxygenation occurred at 30 minutes after any of the 20% reductions from the initial treatment doses of 0, 5, 20, or 80 ppm, except when the final step of discontinuing the gas was reached. The masked, final doses of treatment gas were 0, 1, 4, and 16 ppm. On cessation of the treatment gas, oxygenation decreased for the three groups receiving NO in a dose-related manner. The decrease in oxygenation was statistically and clinically appreciable in the 4- and 16-ppm group but not the 1-ppm group. The decreases in oxygenation on withdrawal of I-NO for treatment successes did not result in any adverse outcomes such as death or need for ECMO.

The data from the present study suggest that I-NO can be safely withdrawn in infants who have improved their oxygenation status and could be classified as treatment successes. Specifically, once the OI is

<10, I-NO can be weaned to 1 ppm without severe reductions in oxygenation in most patients. In a retrospective review of PPHN treatment successes,[12] inhaled N0 was discontinued at 5 ppm. The investigators observed that by increasing the FIO_2 by 0.4, hypoxemia could be avoided. However, the present study indicates that by gradually reducing inhaled NO from the usual treatment dose of 5 to 20 ppm down to 1 ppm before cessation of therapy, substantial compensatory changes in FIO_2 or conventional ventilation may be unnecessary. There was 11 ± 23 mm Hg (mean ± SD) reduction in PaO_2 within one-half hour of stopping the treatment from 1 ppm of NO. In other words, most patients had a reduction in PaO_2 of <34 mm Hg (mean 1 SD) or a drop in PaO_2 from a mean of 105 mm Hg (prechange) to 71 (postchange), when the FIO_2 was of 0.5 to 0.6. This reduction in oxygenation could be explained by an ~10% increase in right-to-left shunt[13,14] which could be theoretically prevented by a 20% increase in FIO_2. Cessation of I-NO from higher levels of inhaled NO could lead to a greater hypoxemia that may not be easily correctable by an increase in FIO_2 alone.

Treatment failures occurred in 34% of placebo and 25% of the pooled I-NO group at 10 ± 11 and 10 ± 13 hours, respectively (mean ± SD). At the time of treatment failure, patients were near ECMO criteria, with oxygenation indices of ~40. Then, investigators took usually <12 hours to wean off the treatment gas, with a trend toward faster weaning for placebo. In contrast to patient successes, there was only fair compliance to completing the withdrawal protocol for patients declared as treatment failures. At this critical stage of PPHN, in which protocol considerations were outweighed by patient interests and preparing rescue management, less than half of the requested data were captured. Accordingly, only a single comparison was made between patient data for placebo and the pooled NO groups.

For treatment failure patients, the OI decreased 13% for the placebo (not significant), but increased 42% ($P = .03$) for the pooled NO group. There was no statistical difference in death or ECMO for treatment failures in the placebo versus the pooled NO group. Because a majority of patients were treated at ECMO centers it was unlikely that an increase in deaths would have occurred. Initially investigators reported no serious adverse events related to NO**.** For treatment failures there were 4 deaths in the pooled NO group and no deaths in the placebo group. This was not a statistically significant increase because patients were initially randomized to NO versus placebo in a 3-to-1 ratio. One patient had severe meconium aspiration syndrome and was declared a treatment failure because of hypoxemia after 56 hours on treatment gas. On cessation of the treatment gas, worsening hypoxemia led to the need for high-frequency oscillation and positive pressure ventilation by hand. A pneumothorax occurred and was treated, but the infant died 1.5 hours after stopping NO. This patient received I-NO at 20 ppm, decreased in 20% decrements throughout 13 minutes.

The present study did not address the causes for the decrease in oxygenation on withdrawal of I-NO. The dose-related reduction in PaO_2 on cessation could have been simply related to significant shunting or ventilation-perfusion mismatch that was still present when NO was withdrawn. For the treatment failure group, the reduced oxygenation on withdrawal could have been related to a gradual, unrecognizable, positive effect of I-NO on patients who eventually failed beyond several hours after the start of therapy.[15] Other explanations for a decrease in oxygenation include a possible down-regulation of constitutive, endothelial, NO synthase activity.[16] Unopposed vasoconstrictor action[17] on the pulmonary circulation, at the time of withdrawal, is another possibility if the underlying disease is not completely resolved.

Conclusion

In summary, our study has shown withdrawal of I-NO is not problematic for treatment successes if the OI is <10 and the I-NO dose is gradually reduced to 1 ppm before cessation. If this method of weaning I-NO is followed, a 20% increase in FIO_2 at cessation of I-NO should prevent transient hypoxemia. As previously reported,[3] 25% of patients with PPHN will go on to become classified as treatment failures using inhaled NO as an adjunct to conventional ventilation. These patients may have rapid and life-threatening reductions in oxygenation (OI >40) when I-NO is discontinued. Therefore, we recommend that patients with continued deterioration after starting I-NO:

1. be transferred to ECMO centers on I-NO[18] if the OI becomes sustained >25 on conventional ventilation; and
2. if further rescue therapy fails, I-NO should be continued until the patient is on circulatory bypass support.

These guidelines should help avoid setbacks in oxygenation after successful treatment and the potential of peritransport mortality for patients in need of ECMO support in the era of NO as an adjunctive therapy to conventional or high-frequency ventilation.

Abbreviations

PPHN – persistent pulmonary hypertension of the newborn
ECMO – extracorporeal membrane oxygenation
I-NO – inhaled nitric oxide
FIO_2 – fractional inspired oxygenation concentration
PaO_2 – arterial partial pressure of oxygen
SD – standard deviation
OI – oxygenation index.

Early Inhaled Nitric Oxide Therapy in Premature Newborns with Respiratory Failure

NJM-July 2006,
John P. Kinsella, M.D., et al.

ABSTRACT

Background

The safety and efficacy of early, low-dose, prolonged therapy with inhaled nitric oxide in premature newborns with respiratory failure are uncertain.

Methods

We performed a multicenter, randomized trial involving 793 newborns who were 34 weeks of gestational age or less and had respiratory failure requiring mechanical ventilation. Newborns were randomly assigned to receive either inhaled nitric oxide (5 ppm) or placebo gas for 21 days or until extubation, with stratification according to birth weight (500 to 749 g, 750 to 999 g, or 1000 to 1250 g). The primary efficacy outcome was a composite of death or bronchopulmonary dysplasia at 36 weeks of postmenstrual age. Secondary safety outcomes included severe intracranial hemorrhage, periventricular leukomalacia, and ventriculomegaly.

Results

Overall, there was no significant difference in the incidence of death or bronchopulmonary dysplasia between patients receiving inhaled nitric oxide and those receiving placebo (71.6 percent vs. 75.3 percent, P=0.24). However, for infants with a birth weight between 1000 and 1250 g, as compared with placebo, inhaled nitric oxide therapy reduced the incidence of bronchopulmonary dysplasia (29.8 percent vs. 59.6 percent); for the cohort overall, such treatment reduced the combined end point of intracranial hemorrhage, periventricular leukomalacia, or ventriculomegaly (17.5 percent vs. 23.9 percent, P=0.03) and of periventricular leukomalacia alone (5.2 percent vs. 9.0 percent, P=0.048). Inhaled nitric oxide therapy did not increase the incidence of pulmonary hemorrhage or other adverse events.

Conclusions

Among premature newborns with respiratory failure, low-dose inhaled nitric oxide did not reduce the overall incidence of bronchopulmonary dysplasia, except among infants with a birth weight of at least 1000 g, but it did reduce the overall risk of brain injury. (ClinicalTrials.gov number, NCT00006401)

Early Inhaled Nitric Oxide Therapy for Term and Near Term Newborn Infants with Hypoxic Respiratory Failure: Neurodevelopmental Follow-Up

J. Pediatrics—March, 2007

Objective

To report the neurodevelopmental outcome of infants enrolled in a randomized multi-center trial of early inhaled nitric oxide (iNO) in term and near term neonates with hypoxic respiratory failure and pulmonary hypertension.

Study design

Neonates born at ≥34 weeks gestation were randomized to early iNO or control group if they required assisted ventilation and had an oxygenation index (OI) ≥15 and <25. A comprehensive neuro-developmental assessment of survivors was performed at 18–24 months of age.

Results

The trial enrolled 299 infants of which 266 (89%) survived to 18–24 months of age (136 - early iNO group and 130 - control group). Follow-up evaluations were done on 234 (88%) of surviving infants. There were no differences between the two groups in the incidence of neuro-developmental impairments (early iNO 27% and control 25%) and hearing impairment (early iNO 23% and control 24%). Mental development index scores were similar for the 2 groups; however, psychomotor developmental index scores were significantly higher for control group (early iNO, 89±17.7 and control, 93.5±18.4).

Conclusion

Early iNO therapy for hypoxic respiratory failure in term and near term infants is not associated with an increase in neuro-developmental impairments or hearing loss at 18–24 months postnatal age.

Inhaled nitric oxide (iNO) therapy reduces the use of extracorporeal membrane oxygenation (ECMO) in term and near term infants with hypoxic respiratory failure (1–4). Based on the initial randomized clinical trials, iNO therapy is commonly used for treating moderate to severe neonatal respiratory failure with an oxygenation index (OI) ≥25 (5). A review of the previous randomized trials (1–4) indicated that initiation of iNO therapy at a lower oxygenation index was associated with lower ECMO use/mortality. Therefore, we conducted a randomized, multi-center clinical trial of early initiation of iNO therapy for babies presenting with respiratory failure at an oxygenation index of 15–25 over a three-year period from July 1998 – May 2001. The primary hypothesis for this study was that initiation of iNO at an oxygenation index of 15–25 compared to use of standard iNO therapy at an oxygenation index ≥25 would decrease the rate of ECMO/mortality from 35% to 20%. A

secondary hypothesis for this study was that early iNO therapy would not increase neurodevelopmental impairment or hearing loss rates among surviving infants at 18–24 months of age compared to use of standard iNO therapy. Analysis of the outcomes observed prior to discharge from the hospital indicated that early iNO therapy did not reduce the combined incidence of ECMO/mortality and the rates of ECMO and mortality individually were similar between the groups. Early iNO therapy decreased the progression of respiratory failure to an oxygenation index >25 and to an oxygenation index >40. Here we report the results of the neurodevelopmental follow-up of the surviving infants at 18–24 months of postnatal age.

Methods

The study was a prospective, randomized, double masked clinical trial conducted in tertiary care neonatal intensive care units in USA and Canada. The full details of the trial methods were published previously (6).

Patient Population

Any infant delivered at ≥34 weeks of gestation with hypoxic respiratory failure secondary to idiopathic pulmonary hypertension, respiratory distress syndrome, perinatal aspiration syndrome, pneumonia/sepsis, or suspected pulmonary hypoplasia was eligible for participation in the trial. Babies were enrolled if they required assisted ventilation with an oxygenation index ≥15 and <25 and an $FiO_2 \geq 0.8$ on any two arterial blood gases in a fifteen minute to twelve hour window.

Infants were excluded from the trial if they were >14 days of postnatal age, had life-threatening congenital malformations, structural heart disease other than patent ductus arteriosus or patent foramen ovale, congenital diaphragmatic hernia, or prior exposure to iNO therapy. Informed consent was obtained from parents/guardians before randomization and all the participating centers obtained approval for the study from institutional review boards. The consent form included a plan to obtain detailed neurodevelopmental and hearing assessments at 18–24 months of postnatal age for surviving infants in the study.

Randomization

Infants were stratified by the study center and were randomized to early iNO or to simulated initiation of early iNO. This was done by a central computer accessed by telephone according to a permuted block design developed and implemented by the data-coordinating center.

Follow-up assessment

Surviving infants were scheduled to be seen at 18-24 months for a complete history, physical exam, audiologic assessment, neurologic

evaluation and developmental testing using Bayley Scales of Infant Development (7). Anthropometric measurements were obtained at the follow-up visit and growth percentiles were plotted using NCHS data. Information about intervening medical problems and socioeconomic data were also collected. The neurologic assessment and developmental evaluations were performed by certified examiners who were trained to reliability in the examination procedure and were masked to study group assignment. The neurologic evaluation was based on the Amiel-Tison neurologic assessment (8) and included an evaluation of tone, strength, reflexes, and posture. Cerebral palsy was defined as abnormal muscle tone in at least one extremity and abnormal control of movement and posture. Cerebral palsy was then classified as mild, moderate, or severe.

Mild cerebral palsy was motor function that interfered slightly with, but did not prevent age appropriate motor activities. Mild cerebral palsy group included babies that are capable of non-fluent walking, asymmetric walking, or persistent toe walking with tight Achilles tendon resulting from increased tone; these children did not require an assistive device for walking. Moderate cerebral palsy was defined as impairment of motor function that interferes with age appropriate motor activities and was associated with ambulation requiring an assistive device or no ambulation; but the child can sit independently or sit with support. Severe cerebral palsy group included children with impairment of function interfering with all age appropriate motor activity to the point that the child was unable to ambulate, sit or have supported sitting. For developmental assessment, the Bayley Scales of Infant Development II (7) were administered and from this information, a mental developmental index (MDI) and psychomotor developmental index (PDI) were derived. A comprehensive audiologic assessment was done including speech awareness in sound field as well as by bone conduction, warbled pure tone thresholds in sound field at 250 to 4,000 Hz and tympanometry. Responses were compared to previously established norms (9). For the purpose of the study, normal hearing was defined as threshold responses to speech awareness in sound field and pure tone thresholds in sound field at ≤40 decibels. Children were classified into four groups: normal hearing, sensory-neural hearing loss, conductive loss, or undetermined. A diagnosis of blindness was based on an ophthalmologist report of uncorrectable vision ≤20/200 in the better eye. Neuro-developmental impairment was defined as the presence of any of the following: moderate or severe CP, Bayley MDI < 70, Bayley PDI < 70, blindness, or permanent hearing impairment requiring amplification.

Statistical Analysis

Continuous variables were compared using t-tests or Wilcoxon tests for nonparametric data. Discrete variables were compared using Chi-Square tests or by Fisher Exact Test as appropriate. A p value of <0.05 was considered significant. 95% confidence intervals for the differences between continuous and discrete variables were computed. A difference

was considered statistically significant if the 95% CI for the difference did not include 0 (10).

Results

A total of 299 infants were enrolled in the original trial (Table I); 30 infants died before discharge including 13 in the early iNO group and 17 in the control group. Of the 269 infants that survived to discharge from the hospital, 3 additional infants died prior to reaching 18 to 24 months of postnatal age (one in the early iNO group and two in the control group). Of the remaining 266 infants, 234 infants (88%) were seen for follow-up evaluation. This included 121 infants in the early iNO group and 113 infants in the control group.

The neonatal characteristics, including birth weight, gestation, and sex distribution did not differ between groups (Table I). Infants in both groups were evaluated at similar chronologic and adjusted postnatal age (Table I). Although all infants in the early iNO group had received iNO, standard iNO therapy, given at an OI of ≥25, was provided to 38% of the surviving infants in the early iNO group and to 50% of the surviving infants in the control group. The number of infants who received ECMO support was similar in both groups. The two groups were similar for ethnic distribution, maternal marital status, and maternal education (data not shown). Overall, 18% of the mothers completed 10 to 12 years of education, 30% had a high school diploma, and 23% attended college.

Information for post discharge medication use and use of adaptive equipment was collected by a standardized parental questionnaire (Table II). At the time of follow-up evaluation, 35% of the babies in the study were re-hospitalized at least one time. This is similar to 36% re-hospitalization rate previously reported in the follow-up of cohort from NINOS trial (11). Post discharge medications most commonly used included bronchodilators and home oxygen. There were no significant differences between the two groups for any of the medical and community resource needs (Table II). No significant differences in growth measurements were noted between the two groups (data not shown). Approximately 20% of the study infants had weight <10 percentile and 15% of the infants had length and head circumference < 10 percentile.

Approximately 87% of the infants were found to be normal by neurological assessment (Table III). The overall incidence of cerebral palsy and the incidence of moderate to severe cerebral palsy were not different between the two groups. Overall, a normal neurological exam was observed in 84% of the infants in the early iNO group and 91% of the control group of infants. There was no difference between the two groups in the incidence of moderate to severe neurological abnormalities between the two groups.

The developmental assessment with the Bayley Infant Development Scale showed no differences in scores for MDI between the groups. The percent of babies scoring at an MDI <70 were similar between the two groups. The psychomotor developmental index scores (PDI) were significantly higher in the control group. The percent of babies scoring <70 on PDI were similar between the two groups. Reanalyzing the data after excluding the 12 infants with moderate to severe cerebral palsy did not significantly influence the trends in MDI and PDI between the two groups. After the exclusion of 12 infants with moderate to severe cerebral palsy, infants in the early iNO group had mean MDI scores of 85.2 ± 19.9 compared to control group scores of 87.9 ± 18.6 (*p* value 0.26). Approximately 21% of the infants in the early iNO group and 19% of the infants in the control group had MDI <70, after the exclusion of these 12 infants. The PDI scores were 91.3 ± 15.3 for the early INO group and 95.7 ± 15.9 for the control group (*p* value =0.006). The percent with PDI <70 remained similar between the two groups, 6.8% early iNO versus 7% control group (*p* value= 0.95).

Overall, 203 of the 234 infants seen at the follow-up examination had a complete audiological assessment. There was no difference between the two groups for the percent of assessed infants having normal exams. There was no difference between the two groups in the incidence of sensori-neural or conductive hearing loss. The number of babies requiring tympanostomy tube placement were similar between the two groups (9.1% early iNO group, compared to 12.6% in the control group). There was no difference between the two groups in the incidence of unilateral or bilateral vision loss. We found that 72% of the early iNO group and 75% of the control group were free of any neuro-developmental impairments (NDI- moderate or severe cerebral palsy, Bayley MDI < 70, Bayley PDI < 70, blindness, or permanent hearing impairment requiring amplification).
A comparison of the outcomes for the 46 infants in the early iNO group and 57 infants in the control group that progressed to standard INO therapy at OI≥25 demonstrated no differences between the two groups for the percent of infants that had moderate to severe abnormality on neurological assessment. Infants who received standard iNO therapy in the two groups also had similar MDI and PDI scores, hearing loss rates and occurrence of any neurodevelopmental impairment (early iNO group 34% and the control group 26%, P = 0.36). Comparison of the data for 12 infants in each group who progressed to receive ECMO also did not demonstrate differences between the two groups for these variables.

Exposure to any iNO therapy was not associated with increased neurodevelopmental impairments in the 178 infants that had exposure compared to 56 control infants that never had iNO exposure. The MDI scores (84.1±19.8 for iNO exposed and 86.4±22.4 for babies that had no iNO exposure, P=0.36) and PDI scores (90.8±17.2 for iNO exposed and 92.6±20.9 for babies with no iNO exposure, P=0.13) were similar for the

two groups. Similarly neuro-developmental outcome for the 24 infants that received ECMO support (MDI 85.8±23.9 and PDI 92.8±15.3) was similar to the 211 infants without ECMO support (MDI 84.5±20, P=0.66 and PDI 91±18.5, P=0.68). We performed secondary analyses of the data to identify association of neuro-developmental impairments with some adjunctive therapies used during the hospital stay that were previously reported to be risk factors for such impairments (12–17). The use of skeletal muscle relaxants (134 infants with exposure and 95 infants without exposure) was not associated with increased neuro-developmental or hearing impairments (data not shown). The 98 infants (43.7%) exposed to postnatal steroids during hospital stay appeared to have a higher incidence of neuro-developmental impairments (34.7%) than infants that had no exposure (19.8%, $p<0.01$). However, further analysis of the data revealed that infants exposed to postnatal steroids were sicker and were more likely to have received volume expanders, vasopressor support, standard iNO therapy and high frequency oscillation, longer duration of ventilator support and had a higher incidence of chronic lung disease. Multiple Logistic regression analysis model showed that steroid exposure is not an independent risk factor for adverse neuro-developmental outcome; odds ratio for neuro-developmental impairments in the unexposed group was 0.51 with 95% confidence intervals of 0.25–1.01 (P=0.053).

Discussion

The early introduction of iNO therapy for term and near- term infants with a moderate degree of respiratory failure (OI =15–25) improved the oxygenation and decreased the progression to more severe respiratory failure (6). However, early initiation of iNO therapy did not reduce the use of ECMO/mortality in this study. The study infants were followed prospectively to 18–24 months of age to determine if this intervention had any effect on the long-term neurodevelopmental outcome for these infants. Although the study was not powered to detect a pre-specified difference in the neurodevelopmental outcome between these two groups, one of the secondary hypotheses of the study was that early iNO would not increase the incidence of neurodevelopmental abnormalities at 18–24 months of age. Our data show that early iNO and control groups did not differ significantly in the majority of long-term neuro-developmental outcome variables. Although the proportion of babies with medical problems such as the need for home medications, oxygen and ventilator support and abnormal neurological assessment appear higher in the early iNO group, 95% CI show that these differences are not significant. The clinically significant neuro-developmental impairments, such as moderate to severe CP, Bailey MDI and PDI < 70, blindness and hearing loss show equivalent outcomes with p Values ≥0.5 and 95% CI for difference distributed well on both sides of 0.

Three randomized trials of iNO therapy included neurodevelopmental follow-up at 18–24 months of age (11, 18, 19). In these studies, which

included a placebo control group for comparison to iNO, no differences in the neurodevelopmental impairments were noted between the control and treatment groups. The early iNO trial enrolled babies with less severe respiratory failure than the previous trial done by our group (11), and the overall incidence of abnormal neurological exam is 12.8% in this trial compared to 21.5% in our previous trial. Moderate - severe cerebral palsy was noted in 3.8% in this trial compared to 7.6% in our previous trial.

Although our trial enrolled babies with less severe respiratory failure, we observed a high prevalence of the use of supportive therapies such as volume expanders and vasopressors, which were used in over 80% and sedation and analgesia, which were used in 96% of the study infants. (6) In addition, over 60% of the study infants received surfactant therapy and 44% of the infants were tried on high frequency ventilation. Skeletal muscle paralysis was used in 56% of infants. Postnatal steroids were given to 43% of study infants. An association between the use of these therapies and a higher incidence of neurodevelopmental abnormalities and hearing loss was suggested in previous follow-up studies (12–17). We found that the use of skeletal muscle relaxants and postnatal steroids was not associated with neuro-developmental impairments in this study in contrast to the previous studies that reported worse long term outcome with the use of these therapies (12–17).

As part of the study protocol we performed a complete audiologic assessment in the study infants. The overall incidence of hearing loss (24%) was similar to what we observed at the follow-up of infants in the NINOS trial (10). Although there was no difference in the incidence of hearing loss between early iNO and control groups in our study, a relatively high incidence of hearing loss persisted in this cohort of less sick infants. Whether this high hearing loss rate is related to the respiratory failure or the use of adjunctive therapies, such as alkalosis, analgesia and neuromuscular blocking agents (12–15) remains unknown.

We found that the MDI scores from the Bayley assessment were similar between the two groups. However, the two groups differed with respect to their scores on the PDI. This difference in the score persisted even after exclusion of babies that were noted to have moderate to severe cerebral palsy. The MDI and PDI scores showed significant variability with a standard deviation of 18–21 points. In addition, the data were subjected to multiple comparisons that may increase the probability of a type 1 error (10) for the observed difference. However, a possible adverse effect of early iNO in term and near term neonates with respiratory failure can not be excluded from our study. We observed lower PDI scores at the follow-up of the NINOS trial for the iNO group compared to control group, though the difference was not statistically significant. When we compared the PDI scores for babies that progressed to standard INO therapy in both treatment groups in the early iNO trial, the difference in the PDI scores was not significant.

Therefore, early initiation of iNO therapy did not have an adverse effect on the outcome for babies that had progression of their respiratory failure. In addition, iNO therapy itself or ECMO support did not affect the neurodevelopmental outcome compared to infants that did not receive these therapies. However, our sample sizes for babies that did not receive iNO therapy (56 infants) and babies that received ECMO support (24) are small.

Our early iNO and control groups were similar for all measured socioeconomic variables. The two groups experienced similar post-discharge medical needs, including rates of hospital readmission, need for home oxygen, tube feedings, and other medications. Therefore, the neurodevelopmental outcomes in our study subjects were unlikely to be influenced by differences in health status or socioeconomic factors.

In conclusion, early iNO therapy was not associated with an increase in medical, neurodevelopmental, or hearing abnormalities at 18–24 months of age compared to standard use of iNO therapy in a population of term or near-term infants with hypoxic respiratory failure. Even though early iNO therapy decreased the progression of respiratory failure in these infants, this apparent benefit was not associated with a decrease in long-term morbidity. Survivors of neonatal hypoxic respiratory failure remain at a significant risk of neurodevelopmental and hearing deficits and require close monitoring and follow up. Whether these abnormalities are related to underlying disease process or to the postnatal interventions used in these infants remains unknown and requires further investigation.

Now for a more indepth look at nitric oxide in the respiratory system, we present you with the following:

Nitric Oxide in Health and Disease Respiratory System

Abstract

During the past decade a plethora of studies have unravelled the multiple roles of nitric oxide (NO) in airway physiology and pathophysiology. In the respiratory tract, NO is produced by a wide variety of cell types and is generated via oxidation of L-arginine that is catalyzed by the enzyme NO synthase (NOS). NOS exists in three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). NO derived from the constitutive isoforms of NOS (nNOS and eNOS) and other NO-adduct molecules (nitrosothiols) have been shown to be modulators of bronchomotor tone. On the other hand, NO derived from iNOS seems to be a proinflammatory mediator with immunomodulatory effects. The concentration of this molecule in exhaled air is abnormal in

activated states of different inflammatory airway diseases, and its monitoring is potentially a major advance in the management of, e.g., asthma. Finally, the production of NO under oxidative stress conditions secondarily generates strong oxidizing agents (reactive nitrogen species) that may modulate the development of chronic inflammatory airway diseases and/or amplify the inflammatory response. The fundamental mechanisms driving the altered NO bioactivity under pathological conditions still need to be fully clarified, because their regulation provides a novel target in the prevention and treatment of chronic inflammatory diseases of the airways.

I. INTRODUCTION

A. Historical View

The small, light, and simple molecule nitric oxide (NO) was once regarded only as a noxious environmental pollutant in cigarette smoke, smog (317), and the exhaust from motorcars, destroying the ozone layer and causing acid rain (68). This bad reputation of NO changed when in the 1980s several lines of research showed that NO is an essential molecule in the physiology of the human body.

Early studies demonstrated that endothelial cells are able to release a labile factor, named as endothelium-derived relaxing factor (EDRF), that diffuses to the adjacent muscle layer and relaxes it (124) at least in part stimulating the formation of cGMP (359). Similarly, biochemical experiments showed that nitroglycerin elicits blood vessel relaxation after its conversion to NO with the subsequent formation of cGMP (299). Finally, in 1987, the proof that NO was similar to EDRF (190, 331) was provided. Subsequently, the importance of NO and other nitrogen oxides in the regulation of various body functions, including platelet aggregation (357) and neurotransmission (40), emerged. Eventually, this set of observations was honored by the Nobel prize in 1998.

Shortly after the publication of landmark papers proposing EDRF to be NO, several investigators made observations suggesting that nitrogen oxides are relevant to respiratory biology. First, Pepke-Zaba et al. (339) initiated a successful trial using inhaled NO (ppm concentrations) as a selective pulmonary vasodilator. Simultaneously, Gustafsson et al. (156) measured endogenous NO (ppb concentrations) in the exhaled air of humans and other mammals. Working independently, Gustafsson's group (345) and three other groups (12, 130, 236) found that NO concentrations were higher than normal in patients with asthma, but low in patients with cystic fibrosis; there was great excitement when these parallel findings were reported at the Biology of Nitric Oxide meeting in Cologne in 1992.

The increased NO levels in exhaled air of asthmatic patients might be explained by an overexpression of the enzyme that synthesizes NO (162,

242). NO can be produced by a number of cells in the airways such as endo- and epithelial cells and inflammatory cells. However, these data regarding endogenous NO in the lung represented a series of paradoxes. For example, how could the alveolar space contain NO if it was thought to "sump" out NO by virtue or hemoglobin reactivity? Or more importantly, why are the concentrations measured in expired air three log orders lower than those used to decrease pulmonary vascular resistance? A tremendous amount of research has subsequently been devoted to addressing the troubling paradoxes of pulmonary NO biology; however, many questions remained unanswered. As an example, Beall et al. (30) have recently suggested that concentrations of NO as low at 200 ppb may be relevant to subtle regulation of oxygen uptake in the lungs, but no role has been directly demonstrated for NO gas itself at physiological concentrations. In this regard, it has been argued from the time of the first studies in endogenous nitrogen oxide biology that NO itself may not be the only, or indeed the most important, product of NO synthase (NOS) activation relevant to respiratory physiology (126, 307).

In addition, NO acts also as a neurotransmitter of the inhibitory nonadrenergic noncholinergic (NANC) nerves. In human central and peripheral airways in vitro, NO appears to account for the bronchodilator NANC response (32, 92). Therefore, a physiological function of NO in the airways might be dilatation of bronchial smooth muscle. It has been known for more than half a century that nitrates induce bronchial relaxation (143). NO and NO donors relax human airway smooth muscle in vitro (151, 438), and a bronchodilatory effect of inhaled NO was demonstrated in guinea pigs and humans during methacholine-induced bronchoconstriction (85, 210).

The other way around, inhibition of NO formation increases airway responsiveness to contractile agents in animals and asthmatic patients (315, 365). Again, we face a paradox in pulmonary nitrogen oxide biology here: although the concentrations of exhaled NO are increased in patients with asthma, airway responsiveness is increased instead of suppressed. During the last few years several studies have been performed to assess the relationship between levels of exhaled NO and lung function parameters or other markers of airway inflammation.

B. Bioactive Forms of NO

NO itself has a short half-life in vivo (1–5 s) because of its reactivity with hemoglobin (223, 266, 419) and a broad spectrum of other biological compounds. It has one unpaired electron, making it a free radical that avidly reacts with other molecules such as oxygen, superoxide radicals, or transition metals. NO may be formed and/or bioactivated as nitroxyl (NO^-) or nitrosonium (NO^+). These chemical species have short half-lives in aqueous solution (<1 s) but are stabilized in biological complexes

with thiols (RS^-... ^+NO), nitrite (O_2N^-... ^+NO), and other targets and intermediates (404). Here, we will refer to NO·, NO^+ and NO^- as "NO", unless specified otherwise. NO is an ubiquitous messenger molecule that affects various biological functions, either at low concentrations as a signal in many physiological processes such as blood flow regulation, platelet reactivity, NANC neurotransmission, and central nervous system memory or at high concentrations as cytotoxic and cytostatic defensive mechanisms against tumors and pathogens (for references, see Ref. 298). Many studies demonstrated a significant role for these nitrogen oxides in modulating pulmonary function and in the pathogenesis of various pulmonary diseases (27, 128, 209). Moreover, NO has been detected in exhaled air of animals and humans (156), and the NO concentrations are changed in different inflammatory diseases of the airways such as asthma (12, 126, 345).

Reactions of NO ultimately lead to the nitration (addition of $-NO_2$), nitrosation (addition of $-NO^+$), and nitrosylation (-NO) of most classes of biomolecules. One of the best known interactions of NO leading to cell signaling is the reversible covalent binding, nitrosylation, with the ferrous heme in soluble guanylyl cyclase. Another aspect of NO signaling are *S*-nitrosothiols (SNO) that appear to be important molecules signaling "NO" bioactivity in the lung. SNOs are products of NOS activation that are present in the airway lining fluid in micromolar concentrations, stored in specific cellular compartments to achieve bioactivity and metabolically regulated to deliver bioactivities both through transnitrosation reactions and through release of NO.

C. Regulation of NOS

NO and related compounds are produced by a wide variety of residential and inflammatory cells in the airways (129). NO itself is generated via a five-electron oxidation of terminal guanidinium nitrogen on the amino acid L-arginine (Fig. 1). The reaction is both oxygen- and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent and yields the coproduct L-citrulline in addition to nitroxyl (NO^-), in a 1:1 stoichiometry (174, 392). The enzyme system responsible for producing NO, first functionally identified in 1990 by Bult et al. (46), is NOS, which exists in three distinct isoforms: *1*) constitutive neuronal NOS (NOS I or nNOS); *2*) inducible NOS (NOS II or iNOS); and *3*) constitutive endothelial NOS (NOS III or eNOS). Protein purification and molecular cloning approaches have identified the three distinct isoforms of NOS. nNOS, iNOS, and eNOS are products of distinct genes located on different human chromosomes (12, 17, and 7 chromosomes, respectively), each with a characteristic pattern of tissue-specific expression (252). All of the three NOS isoforms are expressed in the airways (108, 162, 242, 374, 397).

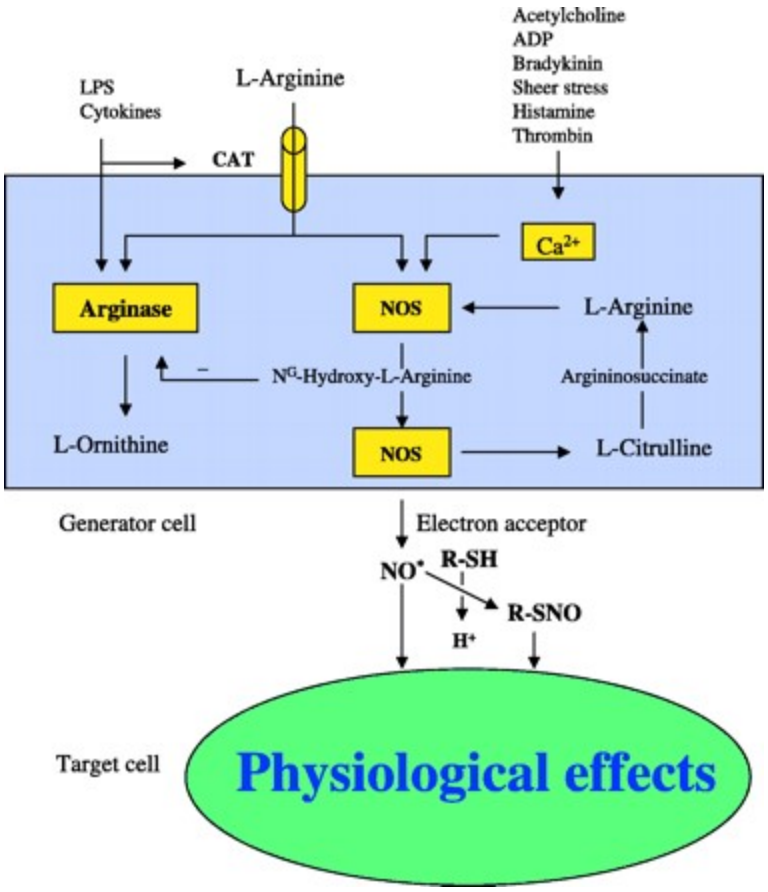

FIG. 1. Simplified over view on L-arginine uptake and metabolism. L-Arginine is transported into the cell via the cationic amino acid transport (CAT) system and can be metabolized by 2 groups of enzymes. Nitric oxide synthase (NOS) converts L-arginine in two steps to nitric oxide (NO) and L-citrulline with *N*^{G}-hydroxy-L-arginine as an intermediate. L-Citrulline can be converted by argininosuccinate to L-arginine. Constitutive (c)NOS is activated by an increase in intracellular Ca^{2+} concentrations. Arginase metabolizes L-arginine to L-ornithine. Lipopolysaccharide (LPS) and several cytokines increases both L-arginine transport and arginase activity. *N*^{G}-hydroxy-L-arginine decreases the arginase activity. NO can bind thiol groups leading to *S*-nitrosothiols (R-SNO). As indicated in the text, both NO and *S*-nitrosothiols have a variety of physiological effects.

Functionally, NOS exists in constitutive (cNOS) and inducible (iNOS) forms (116). cNOS is a Ca^{2+}- and calmodulin-dependent enzyme and releases, within seconds, femtomolar or picomolar concentrations of NO upon receptor stimulation by selective agonists (Fig. 1). iNOS isoform is regulated at a pretranslational level and can be induced by proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α),

interferon-γ (IFN-γ), and interleukin (IL)-1β (303). iNOS releases large quantities (nM concentrations) of proinflammatory NO several hours after exposure, which may continue in a sustained manner (hours or days) (Fig. 2).

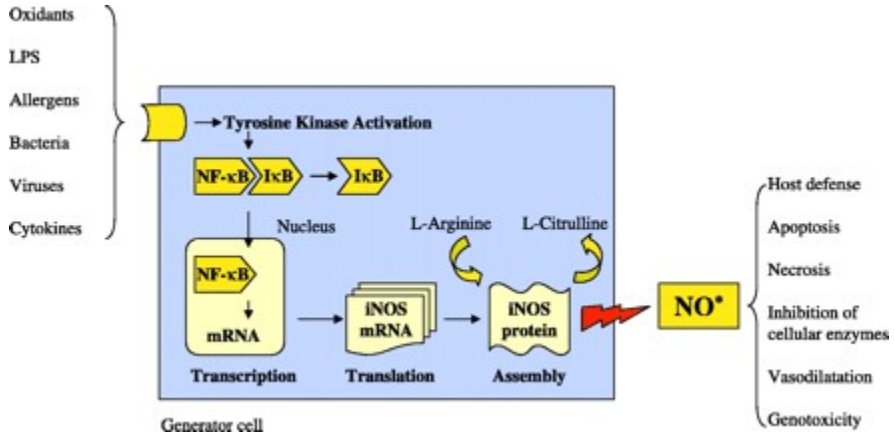

FIG. 2. Overview of the signal transduction pathway leading to the increased expression of inducible nitric oxide synthase (iNOS). A variety of stimuli cause tyrosine kinase activation with subsequent activation of nuclear transcription factor NFκB via phosphorylation and degradation of inhibitory (I)κB. NFκB will accordingly be translocated to the nucleus, and this will lead to mRNA transcription of the iNOS gene. Translation of iNOS mRNA will take place with assembly of the iNOS protein as a result. L-Arginine will be metabolized to L-citrulline and nitric oxide (NO). As described in the text, NO generated by iNOS has beneficial effects (i.e., host defense) but also a number of harmful effects.

The cellular synthesis of the three archetypal enzyme isoforms appears to be dynamically regulated. Changes in NO production are correlated with similar changes in iNOS mRNA abundance, indicating that a major part of iNOS regulation occurs at a pretranslational step such as transcription or mRNA stability (303). Constitutively expressed iNOS in human airway epithelium has been shown by Asano et al. (16) and Guo et al. (154). These latter investigators noted that this unusual expression was lost when human airway epithelium was cultured (154, 155). These authors identified an autocrine mechanism of induction and maintenance of iNOS in human airway epithelial cells through the synthesis and secretion of a soluble mediator (429). Several lines of experimentation have established that transcriptional control mechanisms form an important basis for regulation of this isoform. Induction of macrophage iNOS mRNA by lipopolysacharide (LPS) plus IFN-γ reflects increased iNOS gene transcription without changes in iNOS mRNA stability (303). In marked contrast to the effects of LPS and IFN-γ, transforming growth

factor-β (TGF-β) suppresses macrophage iNOS expression via decreased iNOS mRNA stability and translational efficiency and by decreased stability of iNOS protein, but TGF-β does not alter iNOS transcription (303). Availability of molecular clones corresponding to the mouse iNOS promoter allowed, through the analysis of controlled deletions within the promoter region, the characterization of two major 5'-flanking regulatory regions, one LPS sensitive and the other IFN-γ sensitive, the latter possessing functional characteristics of an enhancer (375). The LPS-sensitive region contains a binding site for NFκB, a transcription factor that has been implicated in the activation of various genes expressed in inflammatory responses. After specific receptor (CD14) stimulation, LPS activates the mitogen-activated protein (MAP) kinase pathway with subsequent activation of NFκB through phosphorylation and degradation of IκB (Fig. 2) (272). Of note, there is evidence for feedback inhibition of this NFκB pathway by NO through two different *S*-nitrosylation pathways (280, 324). An upstream site contains enhancer regions with binding sites for γ-activated site (GAS) element and an IRF-1 specific response element (ISRE) that account for IFN-γ induction (270, 351). IFN-γ is crucial for induction of iNOS expression in airway epithelial cells in vitro (155). IFN-γ signaling to gene expression begins with a specific receptor interaction followed by the Janus kinase (JAK)-STAT1 pathway that involves a tyrosine phosphorylation cascade (164, 172). In fact, pretreatment with genistein, a tyrosine kinase inhibitor, prevents IFN-γ induction of iNOS expression in airway epithelial cells (153). STAT is also able to activate another transcription factor, IRF-1. Both STAT-1 and IRF-1 interact with the response elements GAS and ISRE in the iNOS promoter regions (272, 351).

Whereas transcriptional regulation of iNOS has been established for ~10 years, no expressional regulation was originally known for the other two isoforms. More recent evidence suggests, however, that the expression of nNOS and eNOS can also be regulated under various conditions. nNOS mRNA transcripts and/or protein have been detected in specific neurons of the central and peripheral nervous systems and in nonneuronal cell types such as airway epithelial cells (114). The subcellular localization of nNOS protein varies among the cell types studied. In neurons, both soluble and particulate protein is found. nNOS expression can be dynamically regulated by various physiological and pathological conditions (114). nNOS mRNA upregulation seems to represent a general response of neuronal cells to stress induced by a large array of physical, chemical, and biological agents such as heat, electrical stimulation, light exposure, and allergic substances. Enhanced nNOS expression is often associated with coinduction of transcription factors such as c-*jun* (455) and c-*fos* (422).

While iNOS has been characterized as a soluble (cytosolic) protein, eNOS is targeted to Golgi membranes and plasmalemmal caveolae (small

invaginations in the plasma membrane characterized by the presence of the transmembrane protein caveolin). This complex process is probably dependent on myristoylation, palmitoylation, and tyrosine phosphorylation of the enzyme as well as protein-protein interactions with caveolins (292). In endothelial cells it has been demonstrated that the association between eNOS and caveolin suppresses eNOS activity. After agonist activation the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) promotes calmodulin binding to eNOS and the dissociation of caveolin from eNOS. eNOS-calmodulin complex synthesizes NO until $[Ca^{2+}]_i$ decreases and then the inhibitory eNOS-caveolin complex reforms (292). Interestingly, estrogen upregulates and activates eNOS in endothelial cells. 17β-Estradiol increases NO-dependent dilatation of rat pulmonary arteries and thoracic aorta (142), and estrogen acutely stimulates eNOS in H441 human airway epithelial cells (239). An exciting aspect of this emerging area of study is that estrogen, NO, and caveolae research fields have merged to identify a novel clinical relevant molecular process (468).

D. Localization of NO in the Airways

1. eNOS (NOS III)

Soon after the identification of NO as a messenger molecule generated by endothelial cells, a calcium- and L-arginine-dependent enzyme was identified, and >95% of its activity was sequestered in the particulate fraction of the endothelial (115). Indeed, after the enzyme had been cloned and sequenced (202), and specific antisera for the endothelial isoform of NOS had become available, abundant eNOS immunoreactivity was found in endothelial cells of pulmonary blood vessels. A recent review describes that eNOS is localized to endothelial caveolae by palmitoylation (395).

eNOS is constitutively expressed in human bronchial epithelium (397) and in type II human alveolar epithelial cells (337). Immunoreactivity for eNOS is also localized in the epithelium of human nasal mucosa (219). Ultrastructural studies revealed that eNOS is localized at the basal membrane of ciliary microtubules (458), where it is thought to contribute to the regulation of ciliary beat frequency (197).

2. nNOS (NOS I)

nNOS (NOS I) is localized in airway nerves of humans (78, 106, 152, 242, 271) and animals (77, 78, 152, 242, 271, 428). Substantial species differences are apparent with regard to the extent of innervation and origin of nerve fibers. In human airways, nerve fibers containing nNOS have been shown both by immunohistochemistry and NADPH-diaphorase histochemistry (106, 242, 437). These nerve fibers are present

in the airway smooth muscle, where NO is the major mediator for the neural smooth muscle relaxation (32, 258). The density of these nerve fibers decreases from trachea to small bronchi (106), which is associated with a reduced neural bronchodilatation (92, 437) mediated by the inhibitory NANC (iNANC) system (446). Colocalization with vasoactive intestinal polypeptide (VIP) is frequently observed (250). In human airways, NOS-containing nerve fibers are present around submucosal glands (106), although their functional role for the regulation of glandular secretion is not clear yet. In the lamina propria, NO has potent dilatory effects on blood vessels and on the regulation of plasma extravasation (94).

The cell bodies of these neurons innervating human airways are localized predominantly in the local parasympathetic ganglia (78, 107). Additional sources of NOS immunoreactive nerve fibers are found in vagal sensory and sympathetic ganglia (107, 249, 326). NOS immunoreactive neurons are present in vagal sensory ganglia in humans (50, 107, 249) and in rats (7). In sensory neurons, NO could act as a neuromediator both at the central ending and the periphery (382).

In the central nervous system, reports identified nNOS activity in the cytosolic fraction (114). However, a PDZ-domain has been found in the NH_2-terminal nNOS. [The abbreviation PDZ derives from the first three proteins PSD-95/SAP90, Dlg, and ZO-1 in which these domains were identified (244).] This domain is responsible for the membrane attachment of nNOS through an interaction with the postsynaptic density proteins (PSD) 95 and 93 (42). nNOS is also present in nonneuronal tissues like the respiratory epithelium of guinea pig and rat (94, 242) and in normal endothelial cells (267). In the pulmonary arteries and veins of rats, endothelial cells display immunoreactivity in the cytoplasm (268).

3. iNOS (NOS II)

iNOS (NOS II) has been identified as a separate, calcium-independent isoform, which could be detected in brain, lung, and liver of rats after endotoxin treatment (241). In macrophages it has been revealed by cloning and sequencing that iNOS is expressed de novo at the transcriptional level (273, 456). It is now clear that this isoform is not only localized to macrophages (338), but it can be induced in many different cells (105). In the respiratory tract alone, expression of iNOS has been reported in alveolar type II epithelial cells (440), lung fibroblasts (380), airway and vascular smooth muscle cells (150, 418, 459), airway respiratory epithelial cells (2, 337, 374, 441), mast cells (139) endothelial cells (95), neutrophils (35), and chondrocytes (242). The stimuli that cause transcriptional activation of iNOS in these cells varied widely and included endogenous mediators (such as chemokines and cytokines) as well as exogenous factors such as bacterial toxins, virus infection, allergens, environmental pollutants (ozone, oxidative stress, silica), hypoxia, tumors, etc. (Fig. 2) (140, 462, 464). The expression of iNOS in

the lung can be prevented by glucocorticoids (157). In respiratory epithelial cells of human lung, a "constitutive" expression of iNOS is observed at mRNA (154) and protein level (242). Under normal conditions, however, some investigators could not detect the expression of iNOS (48). It should be stressed, however, that it is difficult to induce iNOS in human cells in vitro and that there are marked differences in the promoter region of iNOS between humans and rodents. Corticosteroids inhibit rodent iNOS, whereas in humans steroids presumably reduce the inflammatory signals that lead to the induction of iNOS.

In conclusion, all three NOS isoforms are localized in the respiratory system (16) where they may cooperatively regulate airway smooth muscle tone and immunologic/inflammatory responses.

E. Arginine Uptake and Metabolism

Because L-arginine is the only physiological substrate for NOS, regulation of L-arginine availability could determine cellular rates of NO production. L-Arginine is an essential amino acid, which is supplied by the diet and actively transported into the cell. L-Arginine displays affinity for the cationic amino acid transporter in various cell types, but the correlation between L-arginine transport and its availability as a substrate for NO synthesis is not well understood (301, 453).

A high-affinity carrier resembling the cationic amino acid transport (CAT) system y^+ is likely to be responsible for the transcellular transport of arginine (Fig. 1), with minor roles being played by systems $b^{o,+}$, $B^{0,+}$, and y^+L (76). The physiological hallmarks of system y^+ are the high affinity for amino acids with a positively charged side chain, its independence from the concentration of extracellular Na^+, and the *trans*-stimulation of arginine transport by the other cationic amino acids L-lysine and L-ornithine. This system has been detected in many cells, among them macrophages, endothelial cells, platelets, and vascular smooth muscle cells (447). System y^+ activity is mediated by the CAT family that is composed of four isoforms, CAT-1, CAT-2A, CAT-2B, and CAT-3 (301). NOS inhibitors based on a modification of the arginine

structure (with a positive charge) are also transported by system y^+. Moreover, arginine itself is a proteinogenic amino acid and, once incorporated into proteins, can be posttranslationally N^G-methylated to the NOS inhibitors N^G-monomethyl-L-arginine (L-NMMA) (exogenous) and asymmetric dimethylarginine (ADMA) (endogenous) or deaminated to form citrulline (447).

The activation of L-arginine transport is sensitive to cycloheximide, demonstrating that de novo protein synthesis is essential for enhanced transporter activity. L-Arginine transport in tissues and many different cell types, such as vascular smooth muscle cells and macrophages, can be

stimulated by LPS, but is hardly affected by TNF-α, IL-1α, or IFN-γ (for an overview, see Ref. 301).

These findings suggest that induction of iNOS and L-arginine transporter activity are dependent on the stimulus used, with an adequate combination of cytokines and/or LPS being responsible for full activation of one or both pathways (Fig. 1). Dexamethasone selectively inhibits the production of NO produced by iNOS whilst having no effect on transport, indicating that the gene for the L-arginine transporter is not sensitive to regulation by glucocorticoids (449). L-Arginine is abundant with a normal dietary intake, but its availability is low owing to extensive protein binding. Oral administration of L-arginine to humans is associated with an increased concentration of NO in exhaled air and was associated with an increase in the concentration of L-arginine and nitrate in plasma (230, 388). These results suggest that an increase in the amount of substrate for NO can increase the formation of endogenous NO.

Arginine can be metabolized by two groups of enzymes. As mentioned above arginine can be converted by NOS to citrulline but can also be catabolized by arginase (Fig. 1).

Arginase exists in two isoforms, liver-type arginase I (165, 220) and nonhepatic type arginase II (36, 302, 435). Arginase I is localized in the cytosol, and arginase II is located in the mitochondrial matrix. iNOS and arginase II are coinduced in LPS-stimulated RAW 264.7 macrophages (304). Moreover, arginase I but not arginase II is coinduced with iNOS in rat peritoneal macrophages and in vivo in rat lung after LPS treatment. In mouse bone marrow-derived macrophages, NOS and arginase activities are regulated by T-helper 1 (Th1) and Th2 cytokines, respectively (297). Moreover, arginase can be induced in the lungs of rats after hyperoxia (355). Allergy is considered to be a Th2-mediated disease, and indeed, arginase activity is increased 3.5-fold in the lungs of guinea pigs after ovalbumin sensitization and challenge (290). Meurs et al. (290) hypothesized that the corresponding airway hyperresponsiveness in these animals is caused by a NO deficiency due to the increased arginase activity (290). Indeed, pretreatment of the tissues with the arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) suppressed the allergen-induced airway hyperresponsiveness (290). Interestingly, N^{G}-hydroxy-L-arginine (NOHA) is an intermediate in the biosynthesis of NO (Fig. 1) (36, 45). LPS-treated rat alveolar macrophages produce high amounts of NOHA (166, 169). The inhibition of arginase by NOHA may ensure sufficient high-output production of NO in activated macrophages, which may be important for the killing of microorganisms. On the other hand, a high production of NO is toxic for cells, and arginase I and mitochondrial arginase II prevent NO-mediated apoptosis in activated macrophages. Therefore, a delicate balance between the beneficial and harmful pathophysiological effects of NO exists in the airways, which might be regulated by arginine metabolism.

F. Molecular Action of NO

NO bioactivities are broadly classified as NO mediated/cGMP dependent and cGMP independent. Many bioactivities, such as airway smooth muscle relaxation, appear to use both. Relaxation of human airway smooth muscle by NO, released as a neurotransmitter, may be partially mediated via cGMP (438). However, airway smooth muscle relaxation to NO and other nitrogen oxides has also been shown to be a cGMP-independent process in humans and a variety of other species (127, 200, 341, 421). cGMP-independent bioactivities, ranging from neurotransmission to cell cycle regulation, appear to involve NO reactivity with alternate metal centers and transfer of an NO^+ (nitrosonium) equivalent from one thiol group to another to up- or downregulate target protein function.

Chemical features of NO radical include its rapid diffusion from the point of synthesis, the ability to permeate cell membranes, the interactions with intracellular molecular sites within both generating and target cells, and its intrinsic instability, all properties that eliminate the need for extracellular NO receptors or targeted NO degradation. The best-characterized target site for NO is the iron bound in the heme component of soluble guanylyl cyclase stimulating conversion of GTP to cGMP and mediating the biological effects attributed to eNOS-derived NO (191). Subsequently, cGMP exerts most of the intracellular actions by coupling to cGMP-dependent protein kinase (PKG). It is generally accepted that cGMP triggers relaxation of smooth muscle by activating two molecular mechanisms: reduction of $[Ca^{2+}]_i$ and reduction of the sensitivity of the contractile system to the Ca^{2+}. The former is due to the ability of activated PKG to phosphorylate several key target proteins with the final effect of $[Ca^{2+}]_i$ reduction. In particular, PKG may stimulate Ca^{2+}-activated K^+ channels (K_{Ca}), inhibit membrane Ca^{2+} channel activity, activate Ca^{2+}-ATPase pump in the plasma membrane and in the sarcoplasmatic reticulum, and inhibit inositol trisphosphate receptor and generation (55). The mechanism of the cGMP-induced Ca^{2+} desensitization is mainly ascribed to the stimulation of myosin light-chain phosphatase activity via inhibition of RhoA-dependent pathway (391). In addition, NO mediates other actions that are independent of guanylyl cyclase and cGMP. The high level of NO released by iNOS has an effect as immune effector molecule in killing tumor cells (170), in halting viral replication (216), and in eliminating various pathogens. In fact, NO has been reported to inhibit the growth of or kill a number of fungi, parasites, and bacteria including *Mycobacterium tuberculosis* (73). This mechanism may involve, at least in part, inhibition of DNA synthesis by inactivation of ribonucleotide reductase and by direct deamination of DNA (251, 451). Finally, NO appears to signal through its reactivity with cysteine groups, particularly those located at consensus motifs for *S*-nitrosylation with

primary sequence or tertiary structure of a protein (Fig. 1) (see below) (340, 405). One of the general mechanisms of antimicrobial defenses involving NO is *S*-nitrosylation by NO of cysteine proteases, which are critical for virulence, or replication of many viruses, bacteria, and parasites (390).

Interaction of NO with many molecular targets also may represent a pathway for its breakdown and inactivation. The most important interaction is probably its reaction with superoxide anion (O_2^-) to yield peroxynitrite anion ($ONOO^-$), which is a potent cytotoxic molecule (356).

G. Regulation of SNO-Mediated Bioactivities

Pulmonary SNO bioactivities are generally those in which functional protein modification is caused by NO transfer to a cysteine thiol (Fig. 1). Specificity of this signaling is achieved by regulation of synthesis, compartmentalization, compositional balance, and catabolism. *S*-nitrosothiol synthesis may be regulated following NOS activation by proteins such as ceruloplasmin, hemoglobin, and albumin (145, 193, 358) and/or NOS itself (144, 392). Specific compartments of relevance are, for example, the mitochondrial intermembrane space, where *S*-nitrosylated caspases are sequestered before being released into the reducing environment of the cytosol and thereby activated by reductive cleavage of the SNO bond (277, 278). Compositional specificity is reflected in the requirement of *S*-nitrosoglutathione (GSNO) to be cleaved to S-nitrosocysteineylglycine, and thereby activated for intracellular transport, by γ-glutamyltranspeptidase (GGT) (18, 261). *S*-nitroso-L-cysteine is highly bioactive in *S*-nitrosylating specific airway epithelial cell proteins, relaxing pulmonary vascular smooth muscle, and increasing neuronal signaling to increased minute ventilation response to hypoxia, in a GGT-independent fashion (261), whereas the D-isomer of *S*-nitrosocysteine (CSNO) is completely nonfunctional in all of these bioactivities (261, 323). Note in this regard that the L- and D-isomers of CSNO release NO at the same rate. Finally, catabolic regulation is exemplified by the activity of gluthatione-dependent formaldehyde dehydrogenase which, by breaking down GSNO to glutathione disulfide (GSSG) and ammonia, regulates cellular levels of *S*-nitrosylated protein (264).

II. NITRIC OXIDE AND PHYSIOLOGY OF THE RESPIRATORY SYSTEM

A. NO and Lung Development

Spatial and temporal nNOS and eNOS expression patterns occur during development of the lung (218, 460). Quantitative developmental studies of mRNA and protein expression as well as immunohistochemical examination revealed that the eNOS isoform increases during fetal

development of the lung (159, 173, 218). In fetal lungs of sheep, eNOS expression was evident in bronchial and proximal epithelia but was absent in terminal and respiratory bronchioles and alveolar epithelium (398). The latter data were confirmed by isoform-specific reverse transcription-polymerase chain reaction assays and NADPH diaphorase histochemistry, which excludes misinterpretation due to immunohistochemistry (64). It was speculated that the rise in fetal lung eNOS may contribute to the marked lung growth and angiogenesis that occurs during the same period of time (333). Shaul et al. (396) suggested that the increase in nNOS and eNOS in the lung early in the third trimester in the primate may enhance airway and parenchymal function in the immediate postnatal period.

B. NO and Transcriptional Regulation in the Lung

S-nitrosylation reactions appear to be of particular relevance to regulation of gene expression in the lung (Fig. 1). Several examples are provided. First, SNOs associated with hemoglobin deoxygenation (261, 335) appear to stabilize the α-subunit of hypoxia-inducible factor 1 (HIF 1) (330) through increased HIF 1 DNA binding activity, in turn increasing downstream expression of hypoxia-inducible genes such as vascular endothelial growth factor in the pulmonary vascular endothelium. Of note, this system only requires SNO formation through hemoglobin deoxygenation rather than the profoundly low oxygen tension, generally <7 mmHg and not relevant in the airway or pulmonary vasculature, required conventionally in vitro (194) to activate HIF 1. Second, physiological levels of GSNO increase DNA binding of gene regulatory protein SP1 and downstream transcription of housekeeping genes such as that for the cystic fibrosis transmembrane regulatory protein (CFTR), while supraphysiological concentrations (>10 μM) completely inhibit SP1 binding, shutting off transcription of housekeeping genes perhaps to redirect cellular resources to stress response. These observations may have relevance to the effect of high levels of nitrosylating agents in the lung, which paradoxically inhibit wild-type CFTR expression at the transcriptional level (467). Third, high levels of nitrosative stress can inhibit NFκB inactivation through direct *S*-nitrosylation or through *S*-nitrosylation of IκB kinase (280, 324). These signaling mechanisms may serve to control cytokine production under physiological conditions, while increasing cytokine production during periods of nitrosative stress.

C. NO and iNANC

Cholinergic and adrenergic systems control the bronchomotor tone together with the NANC system which mediates contraction [excitatory NANC (eNANC)] or relaxation (iNANC) of airway smooth muscle (39, 408). Recent evidence has shown that NO is a neurotransmitter of iNANC system and that nitrergic neurotransmission is present in several organs including the airways (27). Immunostaining studies demonstrated that nNOS is localized into nerves of guinea pig and human airways (242)

which supply vessels, smooth muscle, and lamina propria (108). NOS immunoreactive neurons are found in parasympathetic ganglia and also in sympathetic and sensory (more in jugular than in nodose) ganglia supplying the airways (107, 108). They are more prominent in proximal than in distal airways (437), in agreement with the distribution of iNANC functional responses (108). NO is released from peripheral nerves by nNOS and is activated by calcium entry when the nerve is depolarized (41).

NO mediates approximately one-half of the iNANC (relaxant) response in guinea pig trachea in vitro, and the neuropeptide VIP should be involved in the second half of iNANC relaxant response (258). Of note, VIP-mediated guinea pig airway smooth muscle relaxation is preceded by release of SNOs into the airways (259). The human iNANC response in central and peripheral airways is completely mediated by NO (32, 92). In addition, it has been shown in human airways that iNANC bronchodilator response evoked by electrical field stimulation is associated with a concurrent increase in cGMP content in smooth muscle cells reflecting a cGMP-dependent pathway of neurogenic NO in modulating airway caliber (438). It has also been found that NO-dependent iNANC relaxations are due to the selective activation of K_{Ca} channels in airway smooth muscle (213). NOS may be colocalized with VIP (250, 399), which can also stimulate NO/SNO production (259, 426). The neurons, which release NO, are probably part of the cholinergic pathway. However, stimulation of the preganglionic cervical vagus nerve in an in vitro guinea pig tracheal tube preparation did not cause NO-mediated bronchodilatation, while activation of postganglionic intrinsic nerves provoked bronchodilatation, suggesting that NO-dependent NANC relaxations of the airways are mediated by postganglionic parasympathetic nerves (442). Recently, it has been shown that a NO-dependent component of noncholinergic parasympathetic nerves modulates airway smooth muscle tone at baseline, pointing out the spontaneous activity of noncholinergic nerves during tidal breathing (225). Fischer et al. (104) provided the first evidence that NOS-immunoreactive neurons intrinsic to the guinea pig esophagus project axons to the adjacent trachealis, showing that these neurons could be the postganglionic parasympathetic neurons mediating iNANC relaxation of the trachealis. Furthermore, inhibition of NOS potentiates cholinergic neural bronchoconstriction (31, 439). However, it does not change neural acetylcholine release (31, 439), suggesting that nNOS-derived NO is a functional antagonist to excitatory cholinergic pathway at the postjunctional, and not the prejunctional, level (175).

Physiological and morphological studies of iNANC nerves indicate that they represent a distinct parasympathetic pathway from the well-characterized cholinergic-parasympathetic pathways innervating the airways (52, 175). Consequently, it seems likely that interactions between these nerve pathways occur postjunctionally and are manifested through

their opposing actions on airway smooth muscle. In particular, Canning et al. (51) observed that stimulation of capsaicin-sensitive visceral afferent fibers activates, upon peripheral release of tachykinins, iNANC neurons innervating guinea pig trachealis via activation of both NK_3 and NK_1 receptors (51). It has also been observed that endogenous NO released in association with nerve stimulation regulates the magnitude of eNANC response in guinea pig airways (254). In a recent study it has been observed that the nonadrenergic bronchodilatation induced by capsaicin is suppressed by the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) providing the first evidence of iNANC-derived NO modulation in airway responsiveness of cats in vivo (8).

The fact that in human airways iNANC nerves are the sole neural bronchodilator pathway leads to the hypothesis that any impairment of these nerves such as in inflammatory states has functional consequences for airway patency. Indeed, iNANC responses are significantly reduced from patients with cystic fibrosis, in which there is an intense neutrophilic inflammation of the airways, compared with iNANC responses in normal tissue (27). Furthermore, it has been noted that the circadian variations of the iNANC response may contribute to overnight bronchoconstriction in patients with nocturnal asthma (274). Neural NO-induced relaxation is impaired in guinea pig airways after allergen exposure, without affecting nNOS expression, suggesting a reduced neural NOS activity when allergic inflammation is exacerbated (296).

D. NO and Airway Smooth Muscle Relaxation

The ability of NO to relax smooth muscle has been described in multiple models and muscle types, including airway smooth muscle (55). More than half a century ago, nitrates were supposed to induce bronchial relaxations (143). In 1968 Aviado et al. (19) demonstrated that nebulized nitrovasodilators, but not their administration by intravenous route, reduced baseline lung resistance in anesthetized dogs. However, clinical studies regarding the bronchorelaxant effects of the nitrovasodilators were conflicting (49, 224, 293, 325). Gruetter et al. (151) have shown that nitrovasodilators induce relaxation of isolated airway smooth muscle, activate guanylyl cyclase, and raise cGMP levels. In anesthetized guinea pigs, methacholine-induced bronchoconstriction is reduced by inhaled NO in a concentration-dependent manner from 5 to 300 ppm (85). In addition, a high concentration of NO (300 ppm) causes a small degree of baseline bronchodilatation. Furthermore, in anesthetized and mechanically ventilated rabbits, 80 ppm NO added to the inspired gas prevents increased resistance in response to nebulized methacholine (177). In contrast, there is no effect on pulmonary compliance, suggesting that NO prevented the contraction of the larger airways to a greater extent than the small airways (177). Inhaled NO at a concentration of 80 ppm has no effect in normal human subjects and in chronic obstructive pulmonary disease (COPD) patients, but a small bronchodilator effect in

asthmatic patients (178). NO-dependent airway relaxation is partially due to activation of K_{Ca} channels via guanylyl cyclase and PKG (461). Moreover, these relaxations are due to inhibition of Ca^{2+} release, after stimulation of inositol trisphosphate receptors and ryanodine receptors, from sarcoplasmic reticulum of airway smooth muscle cells mediated via cGMP-dependent mechanisms (214).

Interestingly, there is increasing evidence for another mechanism, in addition to guanylyl cyclase activation, by which NO relaxes human bronchial smooth muscle (24, 127, 199, 341, 421). One of the metabolic pathways for NO also involves its reaction in the presence of thiol to form SNOs (126). SNOs are present in the airways of normal subjects at concentrations sufficient to influence airway tone and have a substantially greater half-life than NO (126). Recently, it has been found that severe asthma is associated with low concentrations of airway SNO, suggesting that the deficiency of such an endogenous bronchodilator mechanism is due to an accelerated degradation of SNO in the lungs of severe asthmatic individuals contributing to severe and refractory bronchospasm (88, 99, 133). Perkins et al. (341) showed that nitrosothiol-induced relaxation is mainly due to cGMP-independent component mediated by reversible oxidation of thiols on unspecified proteins that regulate contraction. Moreover, it has been demonstrated that the activation of K_{Ca} channels mediates part of NO-induced airway smooth muscle relaxation. NO donor-induced relaxation appeared to result in part from a direct cGMP-independent activation of K_{Ca} channels by NO, involving *trans*-nitrosylation reaction that could change the gating of the K_{Ca} channel (1). In a recent study it has also been found in canine tracheal smooth muscle contracted with KCl that GSNO decreases Ca^{2+} sensitivity by affecting the level of regulatory myosin light-chain phosphorylation. This suggests that myosin light-chain kinase is inhibited or that smooth muscle protein phosphatases are activated by GSNO (328). Furthermore, it has been shown that SNO produced a concentration-dependent decrease in ADP-ribosyl cyclase, a regulatory enzyme of $[Ca^{2+}]_i$ in smooth muscle, through a cGMP-independent pathway involving *trans*-nitrosylation mechanisms (445). Finally, it has been examined whether two redox forms of NO, NO^+ (liberated by *S*-nitroso-*N*-acetylpenicillamine) and NO^- (liberated by 3-morpholinosydnonimine) influence the cytosolic concentration of Ca^{2+} and tone of human main stem bronchi. The authors found that NO^+ causes release of internal Ca^{2+} in a cGMP-independent fashion, leading to activation of Ca^{2+}-dependent K^+ channels and relaxations, whereas NO^- relaxes the airways through a cGMP-dependent and Ca^{2+}-independent pathway (200). In conclusion, the endogenous release of NO as well as the exogenous application of NO donors appear to activate several molecular mechanisms that synergically induce airway smooth muscle relaxation.

E. NO Against Airway Smooth Muscle Contraction

1. In vivo studies

Endogenous NO is also able to modulate excitatory airway responses induced by different mediators in animal models. Nijkamp et al. (315) showed in guinea pigs that aerosolized NOS inhibitors enhanced bronchoconstriction induced by increasing intravenous doses of histamine in vivo, suggesting a modulator role for endogenous NO in airway reactivity. Furthermore, Ricciardolo et al. (366) found a L-arginine/NO-dependent modulation of bradykinin-induced bronchoconstriction in guinea pigs that originates independently from the simultaneous activation of the excitatory neural component: postganglionic cholinergic nerves and capsaicin-sensitive afferent nerves (366). The latter group of investigators also noted that acid inhalation in guinea pigs stimulates a tachykinin- and bradykinin-mediated bronchoconstriction that is limited by endogenous release of NO (367). The NK_1 receptor is likely to be responsible for bronchoprotective NO release in the airways after tachykinin stimulation (370). Interestingly, bronchoconstriction provoked by stimulation of protease activated receptor-2 (PAR-2), after intratracheal instillation or intravenous injection of trypsin or the tethered ligands for PAR-2, was inhibited by tachykinin antagonists and potentiated by NOS inhibitor (368). Furthermore, it has been shown that $eNOS^{-/-}$ mice were more hyperresponsive to inhaled methacholine and less sensitive to NOS inhibitor compared with wild-type mice, demonstrating that NO derived from eNOS plays a physiological role in controlling airway reactivity (100). In a recent study airway hyperresponsiveness to methacholine was completely abolished in eNOS-overexpressing, ovalbumin-challenged mice compared with control mice in conjunction with a decrease in the number of lymphocytes and eosinophils in the bronchoalveolar lavage fluid (416). In contrast to eNOS it has also been postulated that in mice nNOS could have a role in promoting airway hyperresponsiveness (74, 75).

Different groups of investigators have shown that acute bronchoconstriction induced by allergen inhalation is potentiated by NOS inhibitors in sensitized guinea pigs in vivo, suggesting a modulation by endogenous protective NO on early asthmatic reaction in animal model (286, 342, 343). Other in vivo studies in guinea pigs have shown that the enhanced airway reactivity induced by allergen (6 h after exposure) is not further potentiated by pretreatment with NOS inhibitors (393, 394) and that virus-induced airway reactivity is completely blocked by low doses of inhaled L-arginine (112), suggesting that allergen- or virus-induced airway hyperreactivity is due to the impairment of endogenous release of protective NO. More specifically, it can be postulated that a deficiency in eNOS-derived NO contributes to the increased airway reactivity after early response (EAR) to allergen (4–6 h), whilst a recovery in iNOS-derived NO production aids the reversal of airway reactivity after the late response (LAR: 24–48 h) in guinea pigs.

This is supposed by the lack of effect of the specific iNOS inhibitor aminoguanidine on airway reactivity to histamine after EAR and by a significant potentiation of the partially reduced airway reactivity to histamine after the LAR induced by inhalation of the specific iNOS inhibitor aminoguanidine (393). More recently, it has been noted that expression of NOS I is reduced at 6 h, but not at 24 h, after allergen challenge in association with a decrease in constitutive NOS activity and in the amounts of exhaled NO. Together with maximal airway hyperresponsiveness to histamine, this suggests that the transient downregulated NOS I may have a role in airway hyperresponsiveness (387). In agreement with the previous studies, Toward and Broadley (423) found that exposure to inhaled LPS initially inhibited NO synthesis and the reduced NO levels coincided with the period of increased airway reactivity to histamine (1 h after exposure) in guinea pig. In contrast, 48 h after LPS exposure, the bronchoconstrictor response to histamine was attenuated (airway hyporesponsiveness) in association with increased levels of NO metabolites in the bronchoalveolar lavage fluid, suggesting a renewal of NO synthesis probably derived by cytokine-induced NFkB activation of iNOS gene (265), with a bronchial relaxant effect. For in vivo studies in humans, the reader is referred to section v*B*.

2. In vitro studies

Bradykinin, endothelin-1, substance P, adenosine, and calcitonin-gene related peptide, applied to the inside of intact tracheal tubes, provoke concentration-dependent relaxations (9, 93, 101–103, 316). The relaxations are reversed into contractions (or contractions are markedly potentiated) by NOS inhibitors, indicating that the relaxant effect in the airways is mediated by the release of endogenous NO (9, 93, 101–103, 316). This effect was mimicked by removal of airway epithelium (111), suggesting that airway epithelium releases NO, which counteracts smooth muscle contraction induced by different spasmogens (9, 93, 101–103, 316). These striking results demonstrate the functional importance of epithelium in airway reactivity, not merely considered as a physical protective barrier between spasmogens and smooth muscle but as a modulator of bronchomotor tone via the release of relaxant substances (so-called epithelium-derived relaxing factors). Treatment of guinea pig trachea in vitro with an inactivator of guanylyl cyclase caused a fivefold increase in the sensitivity to histamine contractile response, indicating the involvement of NO/cGMP pathway in the development of airway hyperresponsiveness (385). Moreover, alterations in guanylyl cyclase activity may account for the strain-related differences in airway reactivity in rats (195). A further study showed that the electrochemical detection of bradykinin-induced NO release in guinea pig airways was fast (duration ~2 s), mainly dependent on the epithelium and absent in Ca^{2+}-free medium, suggesting that a Ca^{2+}-dependent eNOS pathway seems to be involved in the endogenous release of bronchoprotective NO (Fig. 1) (371).

The subsequent step of epithelial-derived NO release is the paracrine effect on airway smooth muscle that is dependent on cGMP increase in the effector cell. In fact, it has been shown that bradykinin raises significantly cGMP levels in guinea pig airways and that this effect is blocked by the pretreatment with NOS inhibitors and in epithelium-denuded preparations. This suggests that cGMP is the final mediator of the bronchoprotection dependent on epithelium-derived NO in this species (102). Meurs et al. (291) demonstrated that polycation-induced airway hyperreactivity to methacholine is dependent on the deficiency of endogenous NO, suggesting that polycationic peptides released by activated eosinophils in the inflamed airways may contribute to the deficiency of bronchoprotective eNOS-derived NO. In a further study these authors found that endogenous arginase activity potentiates methacholine-induced airway constriction by inhibition of NO production in naive guinea pig, presumably by competition with eNOS for the common substrate L-arginine (288). In a recent and elegant study, Ten Broeke et al. (417) showed that calcium-like peptides (CALP1 and CALP2) targeting calcium binding EF hand motif of calcium sensors (calmodulin and calcium channels) may have a role in regulating airway responsiveness by controlling $[Ca^{2+}]_i$ and, consequently, modulating the activity of eNOS (Fig. 1) (417). In fact, they observed that CALP2 inhibition of CALP1-induced airway hyperresponsiveness was Ca^{2+} epithelium dependent and NO mediated (417). Interestingly, they found that bradykinin-induced $[Ca^{2+}]_i$ increase in epithelial cells was markedly higher after incubation with CALP2. In allergen-challenged guinea pigs, the enhanced contractile response to agonists in tracheal preparations after early reaction was not augmented by NOS inhibition as shown in naive animals, suggesting an impairment of protective NO (70). In a further study the same authors showed that L-arginine administration reduced methacholine-induced contraction in isolated perfused tracheas from guinea pigs, indicating that limitation of the substrate may underlie the reduced eNOS activity and the excessive contractile response (69). Finally, it has also been demonstrated that increased arginase activity contributes to allergen-induced deficiency of eNOS-derived NO and airway hyperresponsiveness after early allergen reaction in guinea pigs, presumably by direct competition with eNOS for L-arginine (290).

F. NO and Pulmonary-Bronchial Circulations

1. NO and pulmonary circulation

Nitrogen oxides can account for the biological activity of EDRF and are involved in the regulation of vascular tone (189, 257). Release of NO from endothelial cells in the pulmonary circulation appears to regulate vascular basal tone and counteract hypoxic vasoconstriction (Fig. 1) (344). Furthermore, NO release is apparently decreased in chronic hypoxia (4). Intravenous infusion of the NOS inhibitor L-NMMA increases pulmonary vascular resistance in normal adults pointing towards a role for endogenous NO in the control of pulmonary vascular tone at

baseline (65). In the healthy human, eNOS isoform is present in the endothelium of pulmonary vessels, but its expression is downregulated in patients with primary pulmonary hypertension (136). This suggest that the pulmonary vasoconstriction and the increased smooth muscle layer in the pulmonary vessels, main features of this disease, are associated with impaired expression of eNOS. Interestingly, these abnormalities might be associated with smoking. In a pig model challenge, unfiltered cigarette smoke induced variable responses in the pulmonary circulation, whereas inhalation of filtered smoke caused rapid and consistent pulmonary vasodilatation, probably NO mediated (11). An in vitro study of pulmonary artery endothelial cells incubated with cigarette smoke extract resulted in a time- and dose-dependent decrease in eNOS activity associated with a nonreversible reduction of eNOS protein content and eNOS mRNA. This indicates that chronic exposure of cigarette smoke may contribute to the risk of pulmonary endothelial dysfunction via impairment of eNOS expression (409).

Impaired release of endothelium-derived NO from pulmonary vessels has also been observed in patients with COPD and cystic fibrosis (79). Moreover, isolated pulmonary arteries of patients undergoing heart-lung transplantation for end-stage chronic lung diseases have impaired endothelium-dependent relaxation (67). Recently, it has been demonstrated that overproduction of eNOS-derived NO can inhibit not only the increase in right ventricular systolic pressure associated with pulmonary hypertension, but also remodeling of the pulmonary vasculature and right ventricular hypertrophy induced by chronic hypoxia (Fig. 1) (327). In addition, the lungs of caveolin-1 knock-out mice displayed thickening of alveolar septa caused by uncontrolled endothelial cell proliferation and fibrosis, suggesting an important role for caveolin-1 in endothelium-dependent relaxation of pulmonary vasculature (82). Polymorphisms of the eNOS gene have been associated with high-altitude pulmonary edema, suggesting that a genetic background may underlie the impaired NO synthesis in the pulmonary circulation of this disease contributing to its exaggerated pulmonary hypertension (83).

Interestingly, recent evidence suggests ethyl nitrite is more potent as a selective pulmonary vasodilator in humans and other mammals, and is associated with less withdrawal rebound hypertension, than NO itself (306, 307). This is important because ethyl nitrite is a potent *S*-nitrosylating agent that releases relatively little NO gas. Consistant with recent observations of Gow et al. (144), this observation suggests that the most relevant reaction leading to pulmonary vascular smooth muscle relaxation may involve *S*-nitrosylation chemistry.

2. NO and bronchial circulation

Of note, endogenous NO regulates basal bronchial vascular tone, and exogenous NO accounts for most of the bronchial vasodilatation observed after inhalation of cigarette smoke (11). The airway vasculature

has also been shown to dilate in vivo when animals are ventilated with NO (59). Finally, endogenous endothelial NO significantly influences acetylcholine-induced bronchovascular dilation (389), but not the vagally induced bronchial vascular dilation in sheep (23).

Conflicting results have been reported about the role of endogenous NO in vascular permeability (247). A recent study in guinea pigs demonstrated that NOS inhibitors inhibit airway microvascular plasma leakage induced by substance P and leukotriene D_4 (LTD_4), but not by histamine, suggesting that endogenous NO plays an important role in plasma extravasation induced by some inflammatory mediators (211). The authors also showed that the substance P- and LTD_4-induced rise in plasma extravasation is increased via endogenous NO in the trachea and main bronchi, but not in the intrapulmonay airways, suggesting differential regulation of transvascular protein flux in anatomically different parts of the airway microvasculature. The inhibition of substance P-induced plasma extravasation by NOS inhibitor is possibly due to the vasoconstriction of perfused vessels and the subsequent decrease in local blood flow at the leaky site. It has also been shown that allergen inhalation in sensitized guinea pigs caused microvascular leakage in all airway portions which was suppressed in a dose-dependent manner by pretreatment with the NOS inhibitor L-NAME, suggesting that endogenous NO increases airway microvascular leakage after airway allergic reaction (295). Similar results have been found after administration of LPS, which was able to provoke a significant plasma leakage in rat trachea inhibited by the NOS inhibitor L-NAME. This effect was paralleled by an increase in iNOS activity in LPS animals, suggesting that iNOS-derived NO is responsible for LPS-induced increase in plasma leakage (33). On the contrary, these authors found that in the trachea of vehicle-treated rats L-NAME significantly increased plasma leakage, suggesting an inhibitor role of NO on plasma leakage under physiological conditions. Thus the possibility that alteration of bronchial blood flow by NOS inhibitors confounds the results on plasma leakage cannot be excluded. Further studies examining blood flow through individual microvascular beds would permit greater information about the precise role of endogenous NO on this important aspect of airway microcirculation relevant to disease such as asthma.

G. NO and Mucus-Electrolyte Secretions in the Airways

NOS inhibitors did not affect mucus glycoprotein secretion tonically, but significantly reduced both methacholine- and bradykinin-induced secretion from feline tracheal isolated submucosal glands (312). In addition, NO generator isosorbide dinitrate significantly increased submucosal gland secretion. Taken together, these results suggest that endogenous NO stimulates airway submucosal gland secretion (312). Other secretagogues, such as platelet activating factor, histamine, and TNF-α, enhance release of mucin by guinea pig tracheal epithelial cells,

but the stimulatory effect of each is inhibited by precoincubation of the cells with a competitive inhibitor of NOS. This indicates that these mediators provoke mucin secretion via a mechanism involving intracellular production of NO as a critical signaling molecule (3).

Stimulation of airway bovine epithelial cell ciliary beat frequency by isoproterenol, bradykinin, and substance P is dependent on L-arginine/NO pathway (197). Ciliary motility is an important host defense mechanism of airway epithelium, and it is enhanced by the iNOS inducers alveolar macrophage-derived cytokines, such as TNF-α and IL-1β (198). The cilia stimulatory effect of TNF-α and IL-1β is inhibited by L-NMMA and restored by the addition of L-arginine, suggesting an involvement of iNOS pathway in the regulation of ciliary motility (198). Interestingly, low levels of nasal and exhaled NO in patients with primary ciliary diskinesia (PCD) are related to mucociliary dysfunction, and treatment with NO substrate L-arginine improves mucociliary transport in patients with PCD (269).

Abnormal electrolyte transport produces changes in airway surface liquid volume and composition, inhibits mucociliary clearance, and leads to chronic infection of the airways, as occurs in cystic fibrosis. Modulation of ion channels by NO has emerged recently as a significant determinant of ion channel function (87). NO activates both apical anion channels and basolateral potassium channels via cGMP-dependent pathway (86). Thus NO is a physiological regulator of transepithelial ion movement, and alterations of its generation and action may play an important role in the pathogenesis of lung disorders characterized by hypersecretion of airway surface liquid.

Of note, SNOs have several established effects of potential benefit in the cystic fibrosis airway. These include ventilation-perfusion matching, smooth muscle relaxation, increased ciliary beat frequency, inhibition of amiloride-sensitive sodium transport, augmentation of calcium-dependent chloride transport, augmentation of neutrophil apoptosis, and antimicrobial effects as recently reviewed (403). Additionally, recent evidence suggests that physiological levels of SNOs can increase the expression, maturation, and function of ΔF508 mutant CFTR protein, apparently through *S*-nitrosylation of trafficking proteins involved in the ubiquitination and degradation of the molecule (14, 179, 466). In this regard, it is of particular interest that metabolism of SNOs appears to be accelerated in the cystic fibrosis airway and that SNO levels are nearly undetectable in the bronchoalveolar lavage fluid of patients with mild cystic fibrosis (146). Augmentation of SNO levels by therapeutic administration of GSNO appears to be well-tolerated in patients with cystic fibrosis and to lead to an improvement in oxygenation (403). Of note, inhaled NO does not improve oxygenation in these patients (360).

III. NITRIC OXIDE AND OXIDATIVE STRESS: "NITROSATIVE STRESS"

Reactive oxygen species (ROS) are generated by various enzymatic reactions and chemical processes or they can be directly inhaled. NO can interact with ROS to form other reactive nitrogen species (RNS) (Figs. 2 and 3). ROS, NO, and RNS are essential in many physiological reactions and are important for the killing of invading microorganisms (Fig. 2). However, when airway cells and tissues are exposed to oxidative stress elicited by environmental pollutants, infections, inflammatory reactions, or decreased levels of antioxidants, enhanced levels of ROS and RNS can have a variety of deleterious effects within the airways, thereby inducing several pathophysiological conditions (Fig. 3). ROS and RNS can damage DNA, lipids, proteins, and carbohydrates leading to impaired cellular functions and enhanced inflammatory reactions (Figs. 2 and 3). In this way, ROS and RNS play a prominent role in the pathogenesis of various lung disorders such as adult respiratory distress syndrome (ARDS), interstitial lung disease, cystic fibrosis, COPD, and asthma (37, 110, 141, 182, 362, 431).

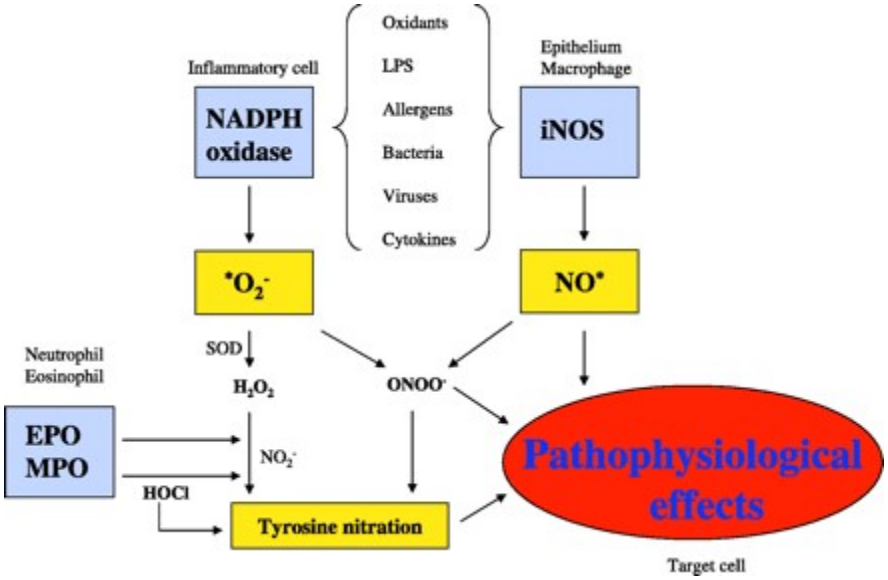

FIG. 3. Schematic overview of how inhaled substances or proinflammatory mediators contribute to the production of reactive oxygen and nitrogen species in the airways that will finally result in pathophysiological effects. Upon appropriate stimulation, inflammatory cells and a number of airway resident cells can generate superoxide (O_2^-) via activation of NADPH oxidase or form high amounts of nitric oxide (NO) via an increased expression of iNOS. NO reacts with superoxide to form the potent oxidant peroxynitrite ($ONOO^-$). Peroxynitrite induces the formation of nitrotyrosine residues; however, tyrosine nitration may also be found after exposure of proteins to nitrite (NO_2^-) in association

with hypochlorous acid (HOCl) and myeloperoxidase (MPO) or eosinophil peroxidase (EPO). As mentioned in the different sections in the text, high concentrations of NO formed by iNOS, peroxynitrite, and tyrosine nitration may all cause a variety of pathophysiological effects.

A. Formation of RNS

Because NO and superoxide are free radicals, both molecules rapidly react with many different molecules in a biological environment. Of particular interest is the interaction between the two molecules and their reactive downstream metabolites. Enhanced cytotoxicity is possible when NO and superoxide are released simultaneously, which is a likely event during inflammatory responses (Fig. 2). For example, the efficient killing of *Salmonella* by murine macrophages is dependent on both NADPH oxidase-derived superoxide and iNOS-derived NO. Many of the products formed by the interaction of superoxide and NO are even more reactive than their precursors. The most direct interaction between NO and superoxide is their rapid isostoichiometric reaction to form the potent oxidant peroxynitrite (Fig. 3) (308, 352). The rate constant of this reaction is near the diffusion controlled limit (4–7 x 10^9 $M^{-1} \cdot s^{-1}$), and the half-life of peroxynitrite at 37°C and pH 7.4 is ~1 s (308, 384). The reaction of peroxynitrite with carbon dioxide is the most important route for degradation of peroxynitrite in biological environments, when carbon dioxide is relatively abundant (430). Many other RNS can emanate from the interaction between NO and superoxide. Besides peroxynitrite formation, NO-derived nitrite can be utilized in the myeloperoxidase pathway leading to NO_2Cl and NO_2 (Fig. 3) (89).

ROS is a collective term that includes a large variety of free oxygen radicals (e.g., superoxide anion and hydroxyl radicals) but also derivatives of oxygen that do not contain unpaired electrons (e.g., hydrogen peroxide, hypochlorous acid, peroxynitrite, and ozone). The univalent reduction of oxygen to superoxide anion is the first step in the formation of ROS. These compounds can either spontaneously or enzymatically dismutate to hydrogen peroxide. Granulocytes contain peroxidases (myeloperoxidase and eosinophil peroxidase) that are able to catalyze the reaction of hydrogen peroxide with halides leading to the formation of hypohalides (e.g., hypochlorous acid, Fig. 3) (22, 240).

Formation of ROS takes place constantly in every cell during normal metabolic processes. Cellular sites for production of ROS include mitochondria, microsomes, and enzymes (e.g., xanthine oxidase, *P*-450 monooxygenase, cyclooxygenase, lipoxygenase, indole amine dioxygenase, monoamine oxidase) (120, 431). Activated phagocytic cells (neutrophils, eosinophils, monocytes, and macrophages) produce large amounts of ROS. These cells are stimulated when encountering inhaled particles, microorganisms, or other mediators that lead to the activation

of the membrane-bound NADPH-oxidase complex and the generation of the superoxide anion (21, 22, 167). Compounds of this enzyme complex have also been found to be present in other cell types such as vascular smooth muscle cells and endothelial cells (205, 279).

NO is a radical molecule that is formed by a wide range of cells, including nerves, (activated) macrophages, fibroblasts, airway and vascular smooth muscle cells, endothelial cells, and epithelial cells (110, 308, 384). In contrast to murine macrophages, it was found that human mononuclear phagocytes did not release large amounts of NO, despite the presence of iNOS (309, 444). However, the lack of NO synthesis in these experiments is probably an in vitro artifact. Adequate stimulation in vivo will lead to NO release by human macrophages (98) and probably cellular interactions (e.g., with airway epithelial cells) and/or local production of regulatory factors are of importance for the NO production (338).

Besides the generation of reactive species via cellular pathways, formation of ROS and RNS in the lungs can also take place after inhalation of exogenous compounds like ozone, nitrogen dioxide, cigarette smoke and other chemicals, and dust particles (246, 431). In addition, such exposures lead to depletion of endogenous antioxidants that are present in the epithelial lining fluid.

Due to the complex chemistry and often short half-life of RNS, the exact metabolic fate in vivo remains unclear. Furthermore, it is almost impossible to attribute a given effect in vivo to a certain reactive intermediate. Nonetheless, some stable end products of RNS are detectable in body fluids and tissues. First, NO decomposes into nitrite and nitrate, and these metabolites can be measured in plasma (222). Furthermore, 3-nitrotyrosine residues have been found in tissue samples by the use of immunohistochemistry (386), but also in biological fluids (322). However, it is often difficult to interpret results from these kinds of experiments since there is a high risk of artifacts. 3-Nitrotyrosine is readily formed by a NO-independent process mediated by myeloperoxidase, with hydrogen peroxide and nitrite as substrates (Fig. 3) (89, 226). Moreover, eosinophil peroxidase is an even stronger promoter of 3-nitrotyrosine formation via this pathway (Fig. 3) (310, 454). At present, the relative contribution of these peroxidase-mediated pathways and peroxynitrite to in vivo 3-nitrotyrosine formation is the subject of debate (90, 361).

Nitrite and nitrate levels in plasma, for example, can reflect the dietary intake rather than NO metabolism in vivo (6). Moreover, NO is also formed enzyme-independently from nitrite under acidic conditions (471). Recently, Hunt et al. (185) showed that the pH in the airways drops dramatically during an acute asthma attack, which facilitates the conversion of nitrite to NO. Hence, increased NO concentrations in the

exhaled air of asthmatic patients may reflect nitrite conversion rather than NOS activity.

Enzymes and chemicals are present within the airway cells and in the airway epithelial lining fluid to protect against the toxicity of generated ROS and RNS. The major enzymatic systems present in the airways are manganese and copper-zinc superoxide dismutases, which rapidly convert the superoxide anion to hydrogen peroxide, catalase that converts hydrogen peroxide into oxygen and water, and the glutathione redox system (GSH-peroxidase and GSH-reductase) that inactivates NO, hydrogen peroxide, and other hydroperoxides (17, 53, 96, 237, 348, 362). The epithelial lining fluid of the respiratory tract contains large amounts of glutathione, and >95% of this glutathione is in the reduced form (54). Moreover, thiol groups in proteins can bind NO. Other nonenzymatic factors with scavenging properties for oxygen radicals that can be present within the airways are vitamin E (α-tocopherol), vitamin C (ascorbic acid), uric acid, β-carotene, flavonoids, taurine, lactoferrin, albumin, and bilirubin. A disadvantage of limiting RNS formation is of course a compromised defense against invading microorganisms. Moreover, nonspecific NOS inhibition may lead to a compromised function of NO as a paracrine messenger, for instance, leading to hypertension (411). The successful use of NOS inhibition is therefore dependent on the isoform of NOS involved, and on the selectivity of the inhibitor used. Nonetheless, limiting superoxide production by NADPH oxidase is of particular interest, since superoxide release is also required for the formation of many RNS, and inhibition of NADPH oxidase should not compromise other NO functions.

B. Airway Damage by "Nitrosative Stress"

The effects of RNS, once formed in vivo, on tissues, cells, and biomolecules are diverse. Important targets of RNS in proteins are, for example, tyrosine residues (432), thiols (125), and heme groups (97). Furthermore, RNS alter lipid oxidation pathways (320), cause DNA damage (470), and inhibit mitochondrial respiration (329). For detailed information about RNS-mediated changes in biomolecules, the reader is referred to an extended review by Eiserich et al. (90). Despite the fact that the exact mechanisms by which RNS affect the function of biological tissues remain unclear, many studies indicate that RNS are able to compromise cell function. Exposure of cells to RNS leads to both apoptosis and necrosis dependent on the severity of cell damage (310). In a recent study it was demonstrated that MAP kinase may mediate signal transduction pathways induced by reactive nitrogen in lung epithelial cells leading to cell death (311). Again, these detrimental effects may affect both an invading pathogen and the (infected) host (Fig. 2).

IV. EXHALED NITRIC OXIDE

Exhaled air of humans contains detectable amounts of NO, in the ppb range as measured by chemiluminescence analyzers (156). The measurement of exhaled NO is critically dependent on expiratory flow (346), which requires careful standardization of the measurement. Such standardization has recently been accomplished by international guidelines on the methods of measurement of exhaled NO, both for adults and in children (13, 26, 227). The levels of NO in the exhaled air are determined by *1*) NO production by various cells in the airways and/or lung parenchyma, *2*) diffusion of NO into the capillary circulation, and *3*) alveolar ventilation and bronchial airflow (187).

Exhaled NO production by the airways and lung parenchyma, in turn, appears to be determined by *1*) the activity of all three NO synthase (NOS) isoforms, but particularly isoforms I and II (75, 443); *2*) the activity of arginase 2 and metabolic enzymes that regulate the endogenous NOS inhibitor asymmetric dimethyl arginine (313); *3*) prokaryotic, denitrifying species colonizing the upper and lower airways (131); *4*) SNO catabolic enzymes (88, 133, 403); and *5*) processes/enzymes that regulate airway pH and nitrite reduction, such as glutaminase (184).

It appears that the NO production and expiratory NO concentrations can be predicted by a two-compartment model of the lung, consisting of a nonexpansible compartment representing the conducting airways and an expansible compartment representing the respiratory bronchioles and alveoli (425). The model predicts that both compartments contribute to NO in the exhaled breath and that the relative contributions of airways and parenchyma can be separated by analysis of the relationship between exhaled NO output (nl/s) against expiratory flow rate (ml/s) (410, 425). Interestingly, such analysis may indeed allow the discrimination of airway diseases, such as asthma, from alveolitis (255) or liver cirrhosis (72) in patients with elevated levels of exhaled NO. This suggests that exhaled NO might be used in differential diagnoses, based on recent theoretical and experimental physiology.

A. Exhaled NO and Bronchial Asthma

Patients with atopic asthma show increased levels of exhaled NO compared with healthy controls (148, 236). In asthma, the increased levels of exhaled NO have a predominant lower airway origin (229, 282) and appear to be associated with increased expression of corticosteroid-sensitive iNOS (386). However, there is recent evidence that exhaled NO levels in asthma are also associated with a known functional missense sequence variant in the eNOS gene (G894T) (407). This indicates that both NOS II and NOS III are important in determining the NO detected in the exhaled air in patients with asthma. Furthermore, exhaled NO may

reflect disease severity (234) and, to a greater extent, clinical control of asthma (402) particularly during exacerbations (71, 231, 281).

Exhaled NO has been used to monitor asthma exacerbations, both spontaneous (281) and induced by steroid reduction (235), and the effect of anti-inflammatory treatment in asthma (234). It can be postulated that asthma treatment with corticosteroids results in a reduction of expired NO levels due to both reducing effects of steroids on the underlying airways inflammation in asthma and inhibitory effects on iNOS expression itself. Oral and inhaled corticosteroids have been shown to result in a rapid (after 6 h following a single corticosteroid treatment) (228) and dose-dependent reduction (203, 433). Since already low doses of inhaled steroids (400 μg budesonide) seem to be sufficient to reduce elevated exhaled NO levels to normal values in patients with intermittent or mild persistent asthma (203), the question arises whether these low NO levels indeed reflect optimal control of the underlying airways inflammation or just switching off of expression of iNOS or of a pH regulatory enzyme such as glutaminase (184). In patients with more severe persistent asthma, airway inflammatory processes may overcome this steroid sensitivity of NO, leading to increased levels of exhaled NO even during treatment with high doses of oral or inhaled corticosteroids (235).

During the last few years several studies have been performed to assess the relationship between levels of exhaled NO and lung function parameters or other markers of airway inflammation. Exhaled NO in patients with asthma is correlated with airway hyperresponsiveness to methacholine (84, 204), as well as peak flow variability (260). Furthermore, exhaled NO is associated with eosinophilic inflammation as determined in blood (401), urine (283), bronchoalveolar lavage (260), and sputum (137) in asthmatics with varying disease severity. Recently, a significant relationship has also been shown between exhaled NO and mucosal eosinophil numbers in bronchial biopsies from children with difficult asthma (336) and from atopic adult asthmatics after allergen challenge (364). This indicates that exhaled NO is a novel noninvasive biomarker reflecting airway eosinophilic inflammation in asthma. High production of endogenous NO such as in acute asthma may result in a deleterious effect and may be involved in the orchestration of eosinophilic inflammation that characterizes asthma.

B. Exhaled NO and Other Respiratory Disorders

Exhaled NO levels in COPD are conflictual (61, 66, 284, 383), but it seems that smoking habits and disease severity are the most important factors influencing exhaled NO levels in these patients (406). Current smokers (232) and severe COPD (particularly in combination with cor pulmonale) (62) show lower levels of exhaled NO than ex-smokers and

mild-moderate COPD. Increased exhaled NO levels have been reported in hospitalized patients during an exacerbation of COPD (5). Interestingly, exhaled NO levels returned to control values only months after discharge of those steroid-treated patients, suggesting different inflammatory mechanisms in COPD compared with the highly steroid-sensitive asthmatics (5). Acidosis, a feature of acute respiratory failure frequently associated with exacerbations of COPD, may also increase the release of NO (185). Moreover, pH is low in exhaled breath condensate during inflammatory diseases (245). Other disorders associated with increased exhaled NO levels include rhinitis (168), bronchiectasis (233), active pulmonary sarcoidosis (300), active fibrosing alveolitis (332), and acute lung allograft rejection (400). In contrast, low levels of exhaled NO have been reported in patients with PCD (215), cystic fibrosis (80, 147), PiZZ phenotype-related α_1-antitrypsin deficiency (275), and pulmonary hypertension (372). Certain pulmonary infections, such as viral respiratory illnesses, increase exhaled NO values (25), while others, such as chronic colonization of the cystic fibrosis airway with denitrifying organsims, attenuate exhaled NO values (131).

In particular, PCD, including Kartagener's syndrome, is a genetic disease characterized by defective motility of cilia, in which the levels of exhaled NO are very low compared with normal subjects. Such low levels of exhaled and nasal NO are not seen in any other condition and are therefore used as a screening procedure to detect PCD among patients with recurrent chest infections or male infertility caused by immotile spermatozoa (47). The mechanism of low NO production by nasal and airway mucosa in PCD is unknown, but it might be linked to genetic abnormalities in iNOS gene expression as in cystic fibrosis (81).

V. NITRIC OXIDE AND PATHOPHYSIOLOGY OF THE RESPIRATORY SYSTEM

A. NO and Immune-Inflammatory Responses in the Airways

1. NO and cytokine networks

In 1991, Jorens et al. (206) showed that pulmonary rat macrophages, alveolar as well as pleural, can produce L-arginine-derived nitrite in a dose- and time-dependent manner, after activation with endotoxin, rat recombinant IFN-γ and opsonized zymosan in vitro (206). The authors found that glucocorticoids blocked the induction of nitrite in alveolar macrophages by all of the stimuli mentioned above (206). These results suggest that part of the anti-inflammatory effect and immunosuppressive effects of glucocorticoids are due to their inhibition of the induction of iNOS. During inflammatory responses a variety of cytokines are expressed and released in the lung and airways. The cytokine network may play an important role in the modulation of inflammation in the local environment. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and muramyl dipeptide, a constituent of the bacterial wall, are able to enhance IFN-γ-induced nitrite production in rat alveolar macrophages in vitro, with GM-CSF serving as a priming factor (207). In addition to alveolar macrophages, rat lung fibroblasts are capable of producing nitrite upon stimulation with IFN-γ. This effect is markedly enhanced in fibroblasts after incubation with endotoxin and IL-1β, suggesting that IL-1β is an efficient priming signal for IFN-γ-induced nitrite production (208). In contrast to NO-inducing cytokines, other cytokines such as transforming growth factor-β, IL-4, and IL-10 have been shown to inhibit the expression of iNOS (314). Recombinant human IL-11 is able to reduce IL-12-induced IFN-γ production and to enhance IL-4 and IL-10 production modulating cytokine production in $CD4^+$ cells with the subsequent reduction in NO production from macrophages (38). Recently, it has been shown that NO inhibited LPS-stimulated inflammatory cytokine production (TNF, IL-1, and MIP-1a), but not basal cytokine levels, by normal human alveolar macrophage, suggesting a modulatory role for NO in proinflammatory cytokine secretion by normal human alveolar macrophage (420).

2. NO and T cells

NO may play a role in nonspecific defense mechanisms against pathogens and may be involved in the signaling between macrophages and T cells (28). $CD4^+$ T helper (Th) cells are important in host defense and have been implicated in chronic inflammatory diseases. Two types of Th cell are differentiated by the pattern of cytokines secreted on activation. Th1 cells release IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, and IL-10 (305, 321). These patterns of cytokine production largely determine the effector functions of the two subsets of T cells (378). Th1 cells produce IFN-γ that activates macrophages to produce NO and kill pathogens (294). Inhibition of NO production by analogs of

L-arginine results in increased susceptibility to parasitic infections, such as those produced by *Leishmania*, mycobacteria, and plasmodium (57, 149, 415). IL-4 secreted by Th2 cells is of critical importance for IgE production and is also involved in the expression of vascular cell adhesion molecule 1 (VCAM-1), which is required for the selective adhesion of eosinophils. The balance between Th1 and Th2 cells determines the outcome of many important diseases. With the use of cloned murine T cell lines, evidence is provided that Th1, but not Th2, cells can be activated by specific antigens to produce large amounts of NO. Furthermore, NO can inhibit the secretion of IL-2 and IFN-γ by Th1 cells but has no effect on IL-4 production by Th2 cells. Thus NO seems to exert a self-regulatory effect on Th1 cells which are implicated in immunopathology (414).

Macrophage-mediated suppression of T-cell proliferative responses to different stimuli involves NO release by alveolar macrophages. In particular, IFN-γ could initiate NO synthesis from macrophages resulting in modulation of lymphocyte proliferation via IFN-γR chains (10). T-cell receptor stimulation induces NO formation triggering programmed cell death (apoptosis) of T cells by a mechanism involving regulation of the expression of FasL (450).

Interestingly, a recent study in mice has demonstrated that in the cytokine milieu of allergic airways inflammation (e.g., IL-4, IL-13) there is increased expression and activity of arginase (469). This suggests that the levels of arginine as a substrate for NOS are reduced in asthma, thereby potentially impairing local NO production. Indeed, in situ hybridization of bronchial biopsy specimens did show expression of arginase I mRNA in the submucosal inflammatory cell infiltrates and bronchial epithelium in patients with asthma, whereas such expression was absent in biopsies from healthy volunteers (469). These observations confirm and extend previous observations by Meurs et al. (290) and may point towards arginase-induced impaired NO synthesis as one of the key mechanisms in the pathophysiology of asthma (434).

3. NO and Th2-mediated inflammation in asthma

NO, derived from airway epithelial cells, macrophages, and Th1 cells, plays an important role in amplifying and perpetuating the Th2 cell-mediated inflammatory response, both in allergic and nonallergic asthma. iNOS may be induced in epithelial cells by exposure to proinflammatory cytokines such as TNF-α and IL-1β secreted by macrophages, and IFN-γ secreted by Th1 cells. It is possible that viral infections may also induce iNOS in airway epithelial cells, augmenting the secretion of NO during asthma exacerbations. With the use of an allergic animal model, it has been shown that the manifestations of allergic airway disease, including infiltration of inflammatory cells (eosinophils), microvascular leakage, and airway occlusion are markedly less severe in the iNOS$^{-/-}$ mutants than in

wild-type animals (457). Interestingly, the suppression of allergic inflammation was accompanied by marked increases in T-cell production of IFN-γ but not by reduction in the secretion of either IL-4 or IL-5. The markedly enhanced production of IFN-γ in $iNOS^{-/-}$ mice was apparently responsible for the suppression of both eosinophils and disease, as in vivo depletion of this factor restored allergic pathology in these animals (457). Thus iNOS promotes allergic inflammation in airways via downregulation of IFN-γ activity and suggest that inhibitors of this molecule may represent a worthwhile therapeutic strategy for allergic diseases including asthma.

In addition, NO has been reported to promote the production of chemotactic factors (chemokines) for eosinophils in mice (424), suggesting the possibility that NO acts as part of a positive-feedback loop in which inflammatory cells produce NO and thereby promote their further recruitment through the action of chemokines.

Recent studies also demonstrated that NO inhibits macrophage-derived IL-12 release, which is a major inducer of Th1 cells, preventing the excessive amplification of Th1 cells (180), and that NO-generating agents increased the secretion of IL-4 in Th2 clones (58). This suggests that despite the complex feedback network regulating NO production, the enhanced IL-4 expression would lead to the expansion of Th2 cells once NO is generated.

B. NO and Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR), which is the main feature of asthma, is defined as an increase in the ease and degree of airway narrowing in response to bronchoconstrictor stimuli. Clinical researchers investigated the capability of endogenous NO to affect AHR in asthma. Ricciardolo et al. (365), for the first time, performed a randomized double-blind placebo-controlled study of the effect of NOS inhibition in bradykinin-induced asthma. The authors described a potentiation of bradykinin- and methacholine-induced AHR after pretreatment with the NOS inhibitor, suggesting a bronchoprotective role for endogenous NO in mild asthma. Furthermore, they found that this potentiation was much greater in AHR to bradykinin compared with methacholine, indicating that a mediator-specific response is involved. In a further study, the same group revealed an impairment of NO synthesis inhibition on AHR to bradykinin in severe asthma, possibly due to the reduction or absence of eNOS in the airway of severe asthmatic patients (363). Following these observations, it has also been discovered that severe asthmatics treated with higher dose of corticosteroids than in the previous study are less hyperresponsive to bradykinin, but that the pretreatment with NOS inhibitor markedly enhanced AHR to bradykinin as shown in mild asthma (34). This suggests an effect of high doses of corticosteroids in renewing eNOS

activity by suppression of iNOS expression. A significant potentiation of NOS inhibitors has also been found in AHR to AMP and histamine, but not to allergen-induced bronchoconstriction in asthmatics (412, 413).

Allergen and viral infection are also called inducers of airway reactivity since they are able to increase native reactivity in animal and human asthma (63, 109). A recent study showed that increased AHR to bradykinin, induced by allergen exposure in asthma, is due to impaired production of bronchoprotective NO, a phenomenon that is associated with downregulation of eNOS and upregulation of iNOS within the airway epithelium (369). The latter findings underscore the relevance of bronchoprotection by endogenous NO to limit AHR in asthma and warrant the development of treatment strategies to restore eNOS activity during exacerbations (369).

Recently, to examine the possible involvement of the eNOS gene as the genetic basis of bronchial asthma it has been investigated whether there was any association between bronchial asthma and polymorphisms of eNOS gene. The study by Lee et al. (253) revealed that the distribution of one genotype (*bb*) of eNOS was significantly higher in the asthma group than in the control population, but the eNOS genotype distribution did not differ significantly among groups of patients with different severities of asthma. In addition, as mentioned above, a recent analysis has demonstrated an association between a missense sequence variant in the eNOS gene and exhaled NO levels in asthma, in the absence of associations of this mutation with the level of airways obstruction or its reversibility in these patients (407). Therefore, all of these results suggest that polymorphisms of the eNOS gene may be associated with the development of asthma, but the severity of asthma may not be influenced by polymorphisms of eNOS gene.

C. NO and Cell Proliferation-Survival in the Airways

1. NO and airway remodeling

Airway smooth muscle hypertrophy and hyperplasia, features of airway remodeling, are important determinants of airway hyperresponsiveness in asthma. In vitro studies have recently demonstrated that DNA synthesis and proliferation of human airway smooth muscle cells (HASMC) are reduced by exogenous administration of NO donors (160, 334). More

recently, it has been demonstrated that NO inhibited HASMC proliferation in G_1 phase via cGMP-dependent pathway, but the inhibition of HASMC proliferation in S phase was due to cGMP-independent inhibition of ribonucleotide reductase (161). These newly discovered antiproliferative effects of NO on airway smooth muscle might become an important clue for future strategies to prevent airway remodeling in chronic asthma and COPD.

2. NO and posttransplant obliterative bronchiolitis

The main cause of mortality following lung transplantation is chronic rejection, manifested morphologically as obliterative bronchiolitis. It has been suggested that damage to the respiratory epithelium initiates proliferation of mesenchymal cells, leading to dense collagenous scarring in small airways. iNOS is strongly expressed in the damaged epithelium in human obliterative bronchiolitis, indicating NO as a mediator of epithelial destruction. Fibroproliferation is associated with changes in morphology of fibroblasts accompanied by alterations in iNOS expression. Taken together, these results suggest a dual role for NO in obliterative bronchiolitis following lung transplantation through destruction of epithelium and stimulation of fibroblast activity (379).

3. NO effects on apoptosis

Nitrogen oxides can promote either cell survival or cell death, depending on the chemical species, redox state, concentration, and target cell type. This paradox was first described by Lipton et al. (262) who showed that physiological concentrations of *S*-nitroso-L-cysteine protected against apoptotic and necrotic neuronal cell death (Fig. 1), while peroxynitrite in similar concentrations caused necrosis, in central nervous system neurons (Figs. 2 and 3). It has been observed that 250 nM (physiological concentrations) of *S*-nitrosocysteinyl glycine protects against eosinophilic apoptosis, while the same concentration of the peroxynitrite donor SIN 1 does not (186). Recently, Nabeyrat et al. (311) showed that MAP kinases may mediate signal transduction pathways induced by peroxynitrite in lung epithelial cells leading to cell death.

Early studies on the effect of nitrogen oxides on cell survival were done with high micromolar or millimolar concentrations of "NO donors," with the idea that NO radical was evolved from these species in nanomolar concentrations and was the only relevant nitrogen oxide. At these high concentrations (all >100 μM), SNOs and NONOates augmented apoptosis in neutrophils (117), macrophages (287) and other leukocytes (43). Several mechanisms might be hypothesized, ranging from protein nitration to nitrosoamine-mediated cytidine to thymidine mutation (451). Of note, Messmer et al. (287) have provided evidence that bcl-2 overexpression protects against this high-dose NO-induced apoptosis. On the other hand, physiological concentration of SNOs protect against apoptosis. The mechanism appears to involve, at least in part, nitrosylation of the active-site cysteine of caspases 3 and 9. This effect was originally described in endothelial cells (176) and later characterized directly in lymphocytes (277). Indeed, there is now evidence that Fas-Fas-ligand binding leads to release of *S*-nitrosylated caspases from the mitochondrial intermembrane space into the cytosol, where these proteases are activated by denitrosylation, leading to apoptosis (278). This picture was recently clarified further by Haendeler et al. (158), who

showed that endogenous or exogenous *S*-nitrosylation of thioredoxin cysteine-69 (which is not one of the redox active cysteines) dramatically increases the antioxidant activity of thioredoxin. This SNO-thioredoxin pool appears to be an important, if not the principal, cellular reservoir of SNO bioactivity. Thus physiological levels of NO/SNO may be antiapoptotic by augmenting antioxidant defenses and by increasing, through transnitrosation reactions, inactivation of caspases. Although there is also evidence for cGMP-dependent upregulation of bcl-2 in lymphocytes (134), it appears that *S*-nitrosylation/transnitrosation reactions represent the principal biochemical mechanism underlying the antiapoptotic effects of physiological levels of nitrogen oxides.

D. NO and Lung Cancer

The role of NO in cancer is multidimensional and based on timing, location, and concentration of the different nitrogen oxides. Several studies have implied that overexpression of NOS in chronic inflammation can lead to genotoxicity, and thus NO is considered a tumor initiating agent (Fig. 2). NO may mediate DNA lesions via formation of carcinogenic nitrosamines, direct DNA mutations or DNA strand breaks by RNS and nitrosation inhibition of systems required to repair DNA lesions mediated by other genotoxic substances such as DNA alkyl transferase and DNA ligase (452). Cigarette smoking, the major cause of lung cancer, contains high concentrations of nitrogen oxides in the gas phase and other oxidants in the tar (465). Recently, it has been shown that incubation of plasmid DNA with extracts of cigarette tar and NO-releasing compound caused synergistic induction of DNA single-strand breakage. This suggest a genotoxic role of RNS, formed by the reaction between NO (gas phase) and ROS formed by autoxidation of polyhydroxyaromatic compounds of the tar, in lung cancer (465). Finally, a recent study noted that an excess of endogenously formed NO may induce a mutation of the p53 tumor suppresser gene containing mainly G:C-to-T:A transversion in the early stage of lung adenocarcinoma (123).

NO may also impact other stages of cancer development. These effects of NO are broad and often self-contradictory, spanning its involvement in cytostatic processes, cellular transformation, formation of neoplastic lesions, and regulation of various aspects of tumor biology (452). Thus NO may have both tumoricidal and tumor-promoting effects. NO has a cytostatic effect on tumor cells through inhibition of cellular respiration via modified activity of mitochondrial aconitase by nitration mechanisms and through inhibition of ribonucleotide reductase suppressing DNA synthesis (Fig. 1). Another possible consequence of NO production in cancer is apoptosis within the growing tumor, and this process has been

implicated in the tumoricidal activity of NO (452). Furthermore, NO derived from leukocytes may have an antitumor role, and in particular monocyte-macrophage series have an important role in host surveillance against cancer (183). The cytotoxic/cytostatic activity of macrophages is, to a great extent, attributed to the upregulation of iNOS. In a recent study the mean fluorescent intensity of iNOS in alveolar macrophage (AM) from patients with primary lung cancer was increased compared with that from controls, and associated with increased exhaled NO levels. This indicates that in primary lung cancer the production of NO from AM is increased as a result of iNOS upregulation (263). Some reports further suggest that NO may contribute to suppression of metastasis. Kong et al. (243) have shown that NO inhibits tumor cell adhesion in a manner similar to the inhibition of leukocyte adhesion described for NO in ischemia-reperfusion injury (248). This suggests that low levels of NO produced by the endothelium will reduce metastasis to tissues such as the lung.

NO has been proposed to be an important mediator of tumor growth, and of note, NO could play an important role in tumor progression via regulation of angiogenesis. Enhanced angiogenesis can lead to accelerated growth of the primary tumor, as well as facilitating the process of metastasis (Fig. 3) (196). Angiogenesis is regulated by the cytokine vascular endothelial growth factor (VEGF) and modulated by a number of other cofactors, including TNF-α and transforming growth factor-β, which in part may also be regulated by NO in lung cancer (15). Paradoxically, other studies indicate that NO may downregulate angiogenesis via inhibition of the transcriptional regulation of the VEGF promoter (452). This paradox has been recently studied to characterize the direct effects of NO at the level of the tumor-endothelium interface with respect to angiogenesis using a Transwell two-compartment culture system with human endothelial cells and two human non-small-cell lung cancers (347). It has been found that baseline component of capillary formation at the endothelial-tumor interface is also NO dependent in line with other observations where endothelial-derived NOS is essential for angiogenesis. However, elevated concentrations of NO in endothelial-tumor microenvironment attenuate capillary formation via downregulation of matrix metalloproteinase activity and inhibition of protein tyrosine phosphorylation in the sprouting tips of nascent capillaries. The extent of inhibition depended on the concentration and flux of NO produced in this milieu (347). Another mechanism by which NO may promote tumor growth is by modulating the production of prostaglandins. In particular, NO increases the production of PGE_2, which may in turn increase the leakiness of tumor vasculature (452). On the other hand, PGE_2 also suppresses NO-dependent macrophage tumoricidal activity. Additionally, permeability of the tumor vasculature is mediated by NO produced by the tumor cells themselves and in turn may facilitate angiogenesis increasing tumor growth.

Finally, it has been found that total NOS activities and the intensity of NOS immunoreactivity are significantly higher in lung adenocarcinoma than those in other types of lung cancers, suggesting a specific role of NO in the metabolism and behavior of lung adenocarcinoma (122).

VI. INHALED NITRIC OXIDE

The purpose of this section is to evaluate the potential use and place of NO inhalation therapy in the treatment of diseases of the respiratory system. To appropriately examine this issue it is important to consider general problems due to the exposure of lung cells to NO in relation to dose and toxicity. Animal studies on the toxicity of inhaled NO (iNO) for up to 6 mo revealed no evidence of side effects using NO doses of <40 ppm (181, 319). Thus proposed treatments with iNO in humans vary from 2 to 36 ppm for periods of a few days to a few weeks (354, 381). NO solubilities from the Ostwald coefficient (448) give equilibrium concentrations of NO in extracellular fluid ranging from 3.2 to 58 nM in the absence of O_2. These concentrations are low, and the loss of NO by autoxidation would be negligible, since autoxidation is second order in NO (256). Thus the fate of iNO should be the following: *1*) loss in exhaled air, *2*) combination with oxyhemoglobin in erythrocytes, and *3*) reaction with O_2^- to form peroxynitrite. The first two possibilities do not exert toxic consequences.

1. In particular, we point out that the expired NO (eNO) values are 3 log orders lower compared with iNO values, suggesting an irrelevant physiological role for eNO.
2. Moreover, iNO reacts for its breakdown by interaction with oxygen and hemoglobin. The rate of the autoxidation with the formation of NO_2^- increases exponentially with the concentration of both oxygen and NO (113). Thus the therapeutic efficacy of iNO may not rise dramatically with increased doses as the more NO given, the faster it is oxidized (238). In fact, higher doses of NO result in a relatively greater proportion of toxic products with little incremental yield of intact NO.
3. Finally, the reaction of NO with O_2^- is extremely rapid (182), and peroxynitrite (ONO_2^-) is toxic at millimolar doses to all types (bacterial and mammalian) of cells. If O_2^- were in great excess to NO, the rate-limiting step in ONO_2^- formation would be diffusion of NO from air into solution. Thus ONO_2^- would approach the number of moles of NO in solution.

When administered as inhaled gas at low concentrations, NO diffuses into pulmonary vasculature of ventilated lung regions and selectively dilates the pulmonary vasculature (118, 121). iNO is distributed predominately to well-ventilated alveoli and not to collapsed or fluid-

filled areas of the lung. Local vasodilation of well-ventilated lung regions will cause a "steal" of pulmonary artery blood flow toward well-ventilated alveoli, improving the matching of ventilation to perfusion and improving arterial oxygenation during acute lung injury. Systemic vasodilation does not occur because of the rapid binding and inactivation of NO by hemoglobin within the circulation (138). This effect is in contrast to that of intravenously administered conventional vasodilators (such as nitroprusside, nitroglycerin, or prostacyclin). These agents nonselectively dilate the pulmonary vasculature and augment blood flow to nonventilated areas, thereby increasing right-to-left shunting and reducing PaO_2.

The first pilot studies in humans have been performed by Higenbottam et al. (171) in 1988 demonstrating that iNO was able to reduce pulmonary hypertension in adult patients without major effects on the systemic circulation (339). A few years later, experiments in animal models revealed that iNO was also able to reverse hypoxic pulmonary vasoconstriction without impairing the pulmonary gas exchange (121, 349). Additionally, Roberts et al. (376) and Kinsella et al. (238) found that iNO might be useful in the therapy of the persistent pulmonary hypertension of the newborn (PPHN). In 1993, Rossaint et al. (381) revealed that both iNO (at doses of 18 and 36 ppm) and infused prostacyclin (4 ng · kg^{-1} · min^{-1}) are able to reduce pulmonary resistance (≈20% fall) in ARDS patients (381). In contrast to prostacyclin, which simultaneously caused systemic hypotension and decreased arterial oxygenation saturation, iNO did not induce any change in systemic hemodynamics, but improved arterial oxygenation significantly. Measurement of ventilation-to-perfusion ratio in these patients showed that intrapulmonary right-to-left shunting was increased by the infusion of prostacyclin but, in contrast, was reduced by iNO at 18 or 36 ppm due to redistribution of pulmonary blood flow toward areas with nearly normal ventilation-to-perfusion ratios. This study did not demonstrate any difference between the two doses of 18 or 36 ppm NO regarding pulmonary resistance and systemic oxygenation.

In ARDS patients, improvement of systemic oxygenation and reduction of pulmonary artery pressure are not correlated during NO dose-response studies. To explain this aspect, two different speculative theories ("diffusion theory" and "kinetic theory") have been postulated. *1*) NO quickly diffuses into tissue reaching a balance between the rate of diffusion and the rate of oxidation or binding to targets. Low doses of iNO probably induce diffusion only into vessels near ventilated alveoli (strictly selective vasodilation in ventilated areas) reducing intrapulmonary shunt areas and increasing systemic oxygenation. High doses of iNO provoke diffusion of the lipophilic NO through the lung tissue reaching nonventilated areas ("shunt areas") and leading to pulmonary vasodilation with further reduction of pulmonary resistance but reversing the beneficial effect on oxygenation. *2*) The kinetic theory is based on the

pharmacokinetic rule that the time to total metabolism of a substance depends on the primary concentration. Pulmonary vasculature system is strictly dichotomous: in particular, from the pulmonary artery up to the final capillaries each vessel divides in two smaller ones without transverse connections and after the capillary system two vessels always rejoin to a larger one, until the pulmonary veins are reached. Thus vessels of shunt areas and of areas with ideal ventilation-to-perfusion ratio are finally united in the pulmonary venous system. Low doses of iNO diffuse into the intravascular space resulting in a low local concentration and acting on local vascular smooth muscle. Low concentration of NO is inactivated by hemoglobin before the venous vessel rejoins with a shunt vessel, thus inducing vasodilation only in the ventilated area. Conversely, high doses of iNO correspond to high intracapillary concentrations. Thus the complete inactivation of NO by binding to hemoglobin requires more time resulting in decreased "afterload" for both ventilated and nonventilated areas, since NO remains partially active after rejoining of the vessels.

The degree of lung inflation may also be an important determinant of the effects of iNO. It has been reported that the recruitment of lung units by application of 10 cmH_2O continuous positive airway pressure (CPAP) augmented the improvement of oxygenation caused by inhaling 40 ppm NO in anesthetized dogs with oleic acid-induced lung injury (353). Application of CPAP reduced shunting regions from 48% of cardiac output to 21% and iNO at 40 ppm selectively reduced pulmonary artery pressure from 30 to 24 mmHg.

The advantage of iNO therapy is the pulmonary selectivity due to the inactivation of NO by its rapid combination with hemoglobin within the pulmonary circulation (373). The disadvantage of this therapy is the short duration of action, since many patients with chronic pulmonary hypertension or severe ARDS require continuous vasodilator therapy. Of note, the toxicity of prolonged NO exposure in humans with acute lung injury is unclear. Because NO is rapidly oxidized to NO_2 in the presence of high oxygen concentrations, the toxic effects of NO_2 may be of concern, especially during prolonged NO inhalation exposures. The effects of the cGMP-specific phosphodiesterase inhibitor Zaprinast on the pulmonary vasodilating effects of iNO in awake, spontaneously breathing lambs with pharmacologically induced pulmonary hypertension (188) have been investigated. The duration of the vasodilator response to iNO was markedly increased by Zaprinast infusion at all the three iNO concentrations. In particular with Zaprinast cotreatment, vasodilation induced by iNO was maintained for 88 min with only 4-min periods of iNO (40 ppm). Finally, the augmentation of the vasodilating effects of iNO by Zaprinast is temporally associated with increased net cGMP release from the pulmonary circulation.

Pulmonary hypertension is a frequent complication of severe COPD and a major cause of morbidity and mortality in this condition (192). Mean pulmonary artery pressure in patients with COPD is usually mild at rest but can rise to abnormally high levels on exercise. Although long-term oxygen therapy improves survival in hypoxemic patients with COPD, it has a negligible effect on pulmonary hemodynamics. Several reports showed that the use of iNO in COPD patients may worsen ventilation/perfusion ratio (V/Q ratio) relationships and exacerbate systemic hypoxemia while lowering pulmonary vascular resistance (135, 217). When NO is delivered to well-ventilated alveolar units with fast time constants, the deleterious impact on gas exchange is avoided (377). Recently, it has been shown that long-term use of pulsed NO with oxygen (where spikes of NO are added at the beginning of inspiration) leads to sustained improvement in pulmonary hemodynamics without worsening hypoxemia in stable COPD patients (436). Benefits of the pulsed method include the reduced formation of nitrogen dioxide and methemoglobinemia. Further studies could shed light whether pulsed NO/oxygen treatment will lead to an improvement in exercise tolerance and survival in patients with hypoxemic COPD.

Finally, in neonates with persistent pulmonary hypertension, low-dose inhaled NO therapy has been shown to lead to a favorable long-term (1 yr) outcome with regard to need of extracorporeal membrane oxygenation without increased incidence of adverse effects (60). As an alternative for iNO, the efficacy has been assessed of inhaled *O*-nitrosoethanol gas (ENO) as a novel alternative means of providing NO bioactivity in the treatment of persistent pulmonary hypertension of newborns. ENO produced sustained improvements in postductal arterial oxygenation and systemic hemodynamics. Increases in methemoglobinemia were modest and toxic NO(x) were not detected. Thus ENO can improve oxygenation and systemic hemodynamics in neonates and seems to reduce rebound hypoxemia and production of toxic by-products (306).
Obviously, the other alternative of inhaled NO is the administration of NO donors (285), such as *S*-nitroso-*N*-acetylpenicillamine or sodium nitroprusside. An interesting development in this area is addition of NO-releasing capacity to well-known drugs, by the ester linkage of an NO-releasing moiety to the conventional drug molecule (221). In this way, various NO-donating drugs, such as NO-prednisolone and NO-releasing nonsteroidal anti-inflammatory drugs (NO-paracetamol, NO-aspirin, salbutamol-nitrate, etc.) are currently under investigation. Presently, these devolepments are still largely taking place outside the respiratory field.

VII. CONCLUSIONS AND FUTURE PERSPECTIVES

During the past 20 years we have witnessed an unforeseen revolution in airway physiology. The discovery of the delicate role of endogenous NO in the homeostasis of various cellular functions and the dynamic behavior of the airways has led to a new, rapidly progressing area of physiological science, which has direct bearing on our understanding of multiple airway diseases. However, we seem to be halfway only. The complexity of NO synthesis and the wide functional profile of its various bioactive forms have not been resolved in full detail yet. Ongoing research in this area will undoubtedly provide novel targets for subtle interventions in the prevention and treatment of airway disease.

Endogenous NO is synthesized by various, independently controlled enzymatic pathways. These can be constitutively expressed as well as induced and regulated at the gene-transcriptional level by several cytokines, chemokines, and mediators. Therefore, NOS is dynamically expressed, in both airway resident cells and infiltrating cells.

The bioactivity of NO is largely provided by *S*-nitrosothiols. However, NO can also be regarded as a free radical that interacts with reactive oxygen species, to form reactive nitrogen species. These include extremely bioactive products such as nitrite, nitrate, nitrotyrosine, and peroxynitrite.

NO has a definite role in gene expression during the embryological development of the airways and lung parenchyma. Based on its various bioactive forms and depending on a wide local concentration range, NO can have either protective or deleterious activities during states of airway damage, inflammation, and repair.

The potentially protective effects of NO include neuromodulation by mediating inhibitory noncholinergic nonadrenergic nerve activity, smooth muscle relaxation, attenuating airway hyperresponsiveness to bronchoconstrictor stimuli, downregulating Th1 cells and their proinflammatory activity, and the killing of invading microorganisms.

The potentially deleterious effects of NO (and reactive nitrogen species) include pro-inflammatory activities, such as vasodilatation and plasma extravasation of the bronchial circulation; increased airway secretions; impaired ciliary motility; promoting Th2 cell-mediated, eosinophilic inflammation; and necrosis and apoptosis (which may also be protective!).

NO is likely to be relevant in the pathogenesis of airway diseases, such as asthma, cystic fibrosis, and COPD. This can either be driven by polymorphisms in NOS genes, or by alterations in NOS gene expression caused by environmental exposure to allergens, cigarette smoke, or respiratory virus infections. The latter exposures appear to result in impaired endogenous protective activity by NO within the airways.

What can we expect during the coming years? It is not surprising that this powerful molecule is a target for drug development. This is supported by the fundamental concept that it seems to be preferable to restore physiological, endogenous inhibitory systems rather than developing unphysiological disease-combating strategies. The success of (inhaled) steroids, as the most effective anti-inflammatory agent in airway diseases, strengthens this view. Needless to say that steroids themselves are strong modulators of NO synthesis, by inhibiting inducible NOS and renewing constitutive NOS activity. What can be expected from the scientists in this field?

Cell biologists will further elucidate the complex synthesis and molecular pathways of NO metabolism, to find the major bioactive compounds and the right targets for intervention.

Geneticists will continue their search for (single nucleotide) polymorphisms in promoter regions and genes of NOS that might be associated with clinical phenotypes of airway disease. This should be expanded by genomic and proteomic approaches using microarray technology, to examine the expression of those genes in individual patients. At present, the development of gene transfer therapy seems to become a realistic approach in the treatment of, i.e., pulmonary hypertension. Recombinant adenovirus overexpressing eNOS (56, 201) or iNOS (44) has been shown to reduce pulmonary vascular resistance and remodeling in animal models of pulmonary hypertension. This approach should also be considered for intervention in other diseases with NO-driven pathophysiology.

Pharmacologists are having novel opportunities to modulate NO synthesis aimed to restore the balance between the protective and deleterious effects of NO. This is potentially beneficial in both airway (29) and alveolar diseases (212). Such interventions might be targeted in various ways, e.g., by using selective iNOS inhibitors (163, 427, 463), NO donors (221), or the above-mentioned usage of NOS gene transfer. In addition, pharmacologists should also explore the potential of arginase inhibitors (e.g., nor-NOHA), to reverse the increase in arginase activity and thereby the attenuation of protective NO activity during allergic inflammation (289, 469). Interestingly, these interventions might be fine-tuned by monitoring NO in exhaled air (276). Obviously, potential adverse effects, such as compromised host defense and pulmonary hypertension in the case of NO synthesis inhibition, should be carefully monitored.

Pathologists should examine the role of NO in modulating airway structure (airway remodeling) in chronic disease states. The antiproliferative effects of NO on airway smooth muscle are very promising in this respect.

Physiologists will further explore the functional role of endogenous NO in regulating airway patency. It cannot be excluded that NO is a major mediator in providing the most potent physiological protection against airway narrowing in healthy human subjects, namely, the bronchodilatation and bronchoprotection after a deep inspiration (119).

Clinicians should further expand their efforts in using exhaled NO as a marker of lung diseases (13). Monitoring adequate fractions of exhaled NO may not only be relevant for airway diseases, but also for parenchymal disease (425).

Hence, NO has already made it from the bench to the bedside, and it is not unlikely that new developments in this area will drastically change respiratory medicine during the coming 5–10 years.

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Final Examination

1. The short-lived vasodilator elaborated by endothelial cells in blood vessels was originally called _____.
    a. NO
    b. NO_2
    c. EDRF
    d. cNOS

2. The source of endogenous nitric oxide is _____.
    a. the upper airway
    b. the lung parenchyma
    c. cNOS activity
    d. not known with certainty

3. Some researchers suggest that low-dose inhaled NO (100 ppb) in ARDS patients might be _____ since the autoinhalation of nasopharyngeal NO is eliminated by endotracheal intubation.
    a. contraindicated
    b. considered replacement therapy
    c. a source of toxicity
    d. unnecessary

4. The increase in cGMP within the cells, induced by NO, _____.
    a. inhibits platelet adherence and aggregation
    b. increases platelet aggregation
    c. increases intracellular calcium
    d. increases the activity of NO in the blood

5. Researchers have found that _____ has the capacity to synthesize NO.
    a. only one specific cell type
    b. no cell type
    c. nearly every cell type
    d. it is impossible to identify which cell type

6. Because nitric oxide diffuses so readily into the blood stream and is inactivated by being bound to hemoglobin, inhaled nitric oxide is _____.
    a. potentially toxic
    b. capable of increasing platelet adherence
    c. non-toxic at high dosage levels
    d. a selective pulmonary vasodilator

7. Which of the following is NOT a biological aspect of nitric oxide?
    a. NO reacts with transition metal ions and with both heme and non-heme metalloproteins.
    b. NO reacts with a variety of biologic metalloproteins including myoglobin and cytochrome oxidase.
    c. NO combines with O_2 to form superoxide (O_2).
    d. Potential by-products of NO metabolism include nitrite (NO_2^-) and nitrate (NO_3^-).

8. Animal studies looking at potential therapeutic effects of inhaled nitric oxide have suggested that it may provide protection against oxidant-induced lung injuries, and that _____.
    a. it has an-ti-inflammatory properties
    b. it has no affect on pulmonary shunt and PVR
    c. it produced significant improvement in P_aO_2 or V/Q as evaluated by MIGET
    d. it should not be used in conjunction with conventional therapies like PEEP or mean airway pressure

9. Which of the following statements is NOT TRUE regarding research findings when using inhaled NO therapy on human subjects with ARDS.
    a. Subjects showed improvement in the ratio of arterial oxygen tension to fractional concentration of inspired oxygen (P_aO_2/F_IO_2) and PAP with no change in systemic hemodynamics when NO was administered by inhalation at 20 ppm.
    b. Subjects with a mean PAP 30 mm Hg were more likely to respond to NO inhalation
    c. Prolonged inhalation of NO resulted in tachyphylaxis.
    d. It is nearly impossible to predict which patients with ARDS will or will not respond to treatment with inhaled NO.

10. Research studies regarding the effects of NO inhalation on survival of patients with ARDS _____.
    a. show significant improvements when dosage is kept at or below 20 ppm
    b. show significant improvements at doses higher than 50 ppm
    c. show the therapy has no impact on survival rates
    d. remain largely unknown

11. Which of the following is NOT one of the three major areas of concern regarding toxicity related to inhaled NO therapy?
    a. nitrogen dioxide (NO_2) production
    b. production of endothelium-derived relaxing factor, or EDRF
    c. methemoglobinemia
    d. production of peroxynitrite

12. In clinical trials utilizing mechanical ventilation systems, researchers have found that the NO_2 concentration is greater with _____.
    a. increased NO concentration
    b. higher F_IO_2
    c. lower VE
    d. all the above

13. In an attempt to counter the potential problem of NO_2 in ventilators, some clinical researchers have tried _____ to remove NO_2.
    a. reducing the amount of O_2 in the concentration
    b. using asbestos filters
    c. using a soda lime absorber in the inspiratory circuit
    d. all the above

14. Production of _____ by NO exposure and the high affinity of hemoglobin for NO explains the selectivity for inhaled NO on the pulmonary vasculature.
    a. NO_2
    b. methemoglobin
    c. nitrates
    d. surfactant

15. Clinical researchers looking into problems associated with the potential for NO conversion to NO_2 in lung units with long residence times have found that when inhaled NO is administered to patients with ARDS, levels of exhaled nitric oxide _____.
    a. are negligible
    b. cannot be measured with any sort of accuracy
    c. of 10-20% of inhaled levels are commonplace
    d. of 50-75% of inhaled levels are commonplace

16. While researchers have yet to discover much about the potential intracellular toxicity of inhaled NO at the doses used with ARDS, it is known that superoxide (O_2^-) reacts with NO to produce _____.
    a. oxyhemoglobinemia
    b. methemoglobinemia
    c. peroxynitrite
    d. alveolar cell hyperplasia

17. Which of the following clinical conditions is NOT considered potentially benefitting from administration of inhaled NO therapy?
    a. Left ventricular failure
    b. Primary pulmonary hypertension
    c. COPD and chronic pulmonary fibrosis
    d. Congenital diaphragmatic hernia

18. The risk of significant NO_2 production at NO doses 20 ppm in adult ventilator systems is not significant unless _____.
    a. the NO has been diluted with O_2
    b. the FIO_2 is high and the minute ventilation is low
    c. a high-concentration NO cylinder is used
    d. the delivery system doesn't include a soda lime canister

19. The disadvantage of systems that inject NO into the ventilator circuit during the inspiratory phase by using a nebulizer-drive mechanism that operates during inspiration, is that _____.
    a. it does not work well with varying inspiratory flow patterns such as pressure control
    b. it cannot deliver a stable dose with ventilatory modes such as pressure support in which tidal volume and inspiratory time vary from breath to breath
    c. precise control of the inspired NO concentration is not possible with this technique
    d. all of the above

20. Measurement of methemoglobin levels is facilitated by the fact that the _____ of methemoglobin differs from that of normal hemoglobin.
    a. light absorption
    b. atomic weight
    c. bonding ability
    d. oxidation rate

21. Researchers report that it is also important to _____ due to the effect of NO/N_2 dilution on delivered FIO_2 because air-O_2 blenders do not always deliver a precise NO concentration.
    a. premix the NO with N_2 (or air)
    b. introduce the mixture proximal to the gas inlet of the ventilator
    c. analyze FIO_2
    d. all the above

22. While nitric oxide itself in not a selective pulmonary vasodilator, however, it becomes one _____.
    a. after being exposed to EDRF
    b. when inhaled
    c. when mixed with O_2
    d. all the above

23. Which of the following is NOT one of the conclusions reached by clinical researchers regarding ideal dosage for inhaled NO therapy?
    a. Several studies have reported effective doses at <5 ppm.
    b. It is appropriate to initiate inhaled NO therapy at a dose of 20 ppm, and if oxygenation improves, decrease the inhaled NO to the lowest effective dose.
    c. Doses commonly used with ARDS (<80 ppm), inhaled NO is considered relatively free of toxicity.
    d. There are no major complications related to inhaled NO, however the potential for complications definitely exists.

24. Treatment of methemoglobinemia normally involves the _____, which increases NADH-methemoglobin reductase, or it can be treated by administration treated with ascorbic acid (vitamin Q).
    a. administration of superoxide (O_2-)
    b. administration of positive end-expiratory pressure (PEEP)
    c. infusion of EDRF
    d. infusion of methylene blue

25. The traditional and well-established method for analyzing NO and NO_2 is _____, a technique originally used for many years in industrial and environmental applications, and more recently adapted to biomedical uses.
    a. electrochemical analysis
    b. chemoluminescence analysis
    c. inspired and expired gas analysis
    d. spectrophotometry (CO-oximetry)