PULMONARY NITRIC OXIDE IN PRETERM AND TERM INFANTS WITH RESPIRATORY FAILURE

OUTI AIKIO

Department of Paediatrics and
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University of Oulu

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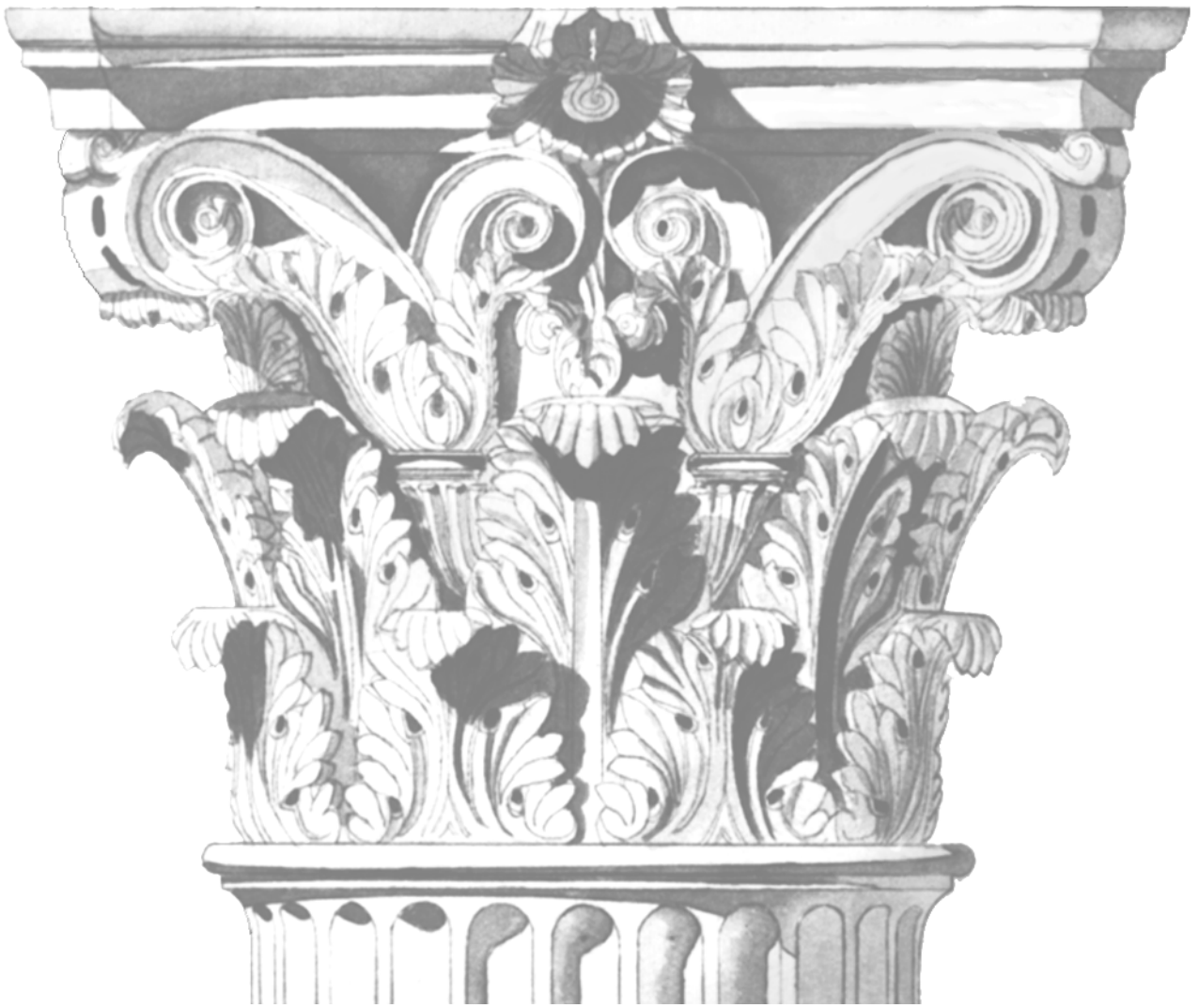

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Abstract

The aim of the study was to evaluate pulmonary endogenous and inhaled nitric oxide (NO) in neonates with severe respiratory failure.

Infant autopsy documents were reviewed for fulminant early-onset bacterial pneumonia. 12 infants with the onset at < 72 h of age and three control groups were identified. Immunohistochemistry revealed that 11 of the infants with early-onset pneumonia (92%) had no or faint inducible nitric oxide synthase (NOS2) staining in their alveolar macrophages (AM). All control infants, regardless of their postnatal age, had NOS2-positive AM. The marker of NO-toxicity, nitrotyrosine, was low in all specimens. To confirm this finding, airway specimens of 21 newborns requiring mechanical ventilation were examined. Seven of them had fulminant early-onset pneumonia with maternal ascending intra-uterine infection (IUI). The controls had no infection at birth despite IUI or neither infection nor IUI. In early-onset pneumonia, NOS2 and nitrotyrosine immunoreactivity were low at birth and increased during the recovery phase ($p < 0.05$). Analyses of interleukin-1β and surfactant protein A showed the same pattern of age-dependent change.

Of the autopsied infants, 12 had received inhaled NO (iNO) before death. Each case was paired with a matched control. Additional five infants without respiratory failure prior to death were also studied. The iNO-treated ones tended to have more intensive NOS2 staining in the bronchiolar epithelium and adjacent tissue than the controls. No differences in other NOS isoforms or nitrotyrosine were detected.

A novel method for exhaled NO measurements of intubated infants was developed. Six preterm and six term newborns were prospectively recruited for expired and nasal NO measurements. During the first week of life, the preterm infants showed a different pattern of exhaled NO excretion compared to the term infants.

For the pilot intervention study on very early iNO, the eligible patients had a birth weight < 1500 g and progressive, therapy-resistant respiratory failure before five hours of age. Five infants received iNO, showed immediately improved oxygenation and survived without deleterious side effects.

Deficient production of NO in small premature infants is associated with severe infection and respiratory failure. Very early iNO therapy may be exceptionally effective in a select group of infants, and did not appear to cause oxidation lung injury.

Keywords: chemiluminescence, infant, nitric oxide, nitric-oxide synthase, nitrotyrosine, respiratory insufficiency

Come away, O human child, to the waters
and the wild,
With a fairy hand in hand,
For the world's more full of weeping
Than you can understand -*W B Yeats*

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Oulu, October 2002 Outi Aikio

Abbreviations

a/A–ratio	arterial-alveolar ratio for oxygen tension
AM	alveolar macrophages
ARDS	acute respiratory distress syndrome
cGMP	cyclic guanosine monophosphate
CI	95 % confidence interval
CLD	chronic lung disease
ECMO	extra-corporeal membrane oxygenation
FiO_2	fraction of inspired oxygen
GBS	group B Streptococcus
IL	interleukin
••••γ	interferon gamma
iNO	inhaled nitric oxide
IUI	intra-uterine infection
HFOV	high-frequency oscillatory ventilation
MAP	mean airway pressure
met-Hb	methaemoglobin
NADPH	nicotinamide-adenine dinucleotide phosphate
NO	nitric oxide
NO_2	nitrogen dioxide
NOS	nitric oxide synthase
NOS1	neuronal nitric oxide synthase
NOS2	inducible nitric oxide synthase
NOS3	endothelial nitric oxide synthase
$OH^{\cdot}$	hydroxyl radical
OI	oxygenation index
$ONOO^{-}$	peroxynitrite anion
OR	Odds ratio
ppb	parts per billion
PPHN	persistent pulmonary hypertension of the newborn
ppm	parts per million
PROM	premature rupture of foetal membranes

RDS	respiratory distress syndrome
SP	surfactant protein
TNF-α	tumour necrosis factor alpha
VI	ventilatory index

List of original papers

This thesis is based on the following articles, which are referred to in the text by their Roman numerals I-IV:

I Aikio O, Vuopala K, Pokela ML, Hallman M (2000) Diminished inducible nitric oxide synthase expression in fulminant early-onset neonatal pneumonia. Pediatrics 105:1013-5.

II Aikio O, Vuopala K, Pokela ML, Andersson S, Hallman M (2003) Nitrotyrosine and NO synthase in infants with respiratory failure – influence of inhaled NO. Pediatr Pulmonol, in press.

III Aikio O, Pokela ML, Hallman M (2002) Exhaled and nasal nitric oxide in mechanically ventilated preterm and term newborns. Acta Paediatr 91:1-9, in press.

IV Aikio O, Saarela T, Pokela ML, Hallman M (2003) Nitric oxide treatment and acute pulmonary inflammatory response in very premature infants with intractable respiratory failure shortly after birth. Acta Paediatr, in press.

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1 Introduction

Severe respiratory failure of a newborn usually requires intensive care, including intubation and mechanical ventilation, surfactant replacement therapy, intravenous use of antibiotics, inotropes and sedation. Despite these conventional therapies, persistent foetal circulation may complicate the respiratory distress. Nitric oxide therapy is a new possibility to treat this life-threatening syndrome.

Nitrogen monoxide (NO, nitric oxide) is one of the smallest molecules found in nature, consisting of one single atom of nitrogen and one atom of oxygen, with a molecular weight of 30 Da (1). Today, it is recognized as a key-signalling molecule in most organ systems, affecting a wide range of biological functions.

In the late 1970s, NO was demonstrated to activate the soluble enzyme guanylate cyclase and to stimulate the accumulation of cyclic guanosine monophosphate (cGMP) in numerous tissues (2). NO was later found to be the mediator of guanylate cyclase activation (3). In 1979, NO was discovered to be a potent vascular smooth muscle relaxant (4) and inhibitor of the human platelet aggregation. Thus, the pharmacological effects and the mechanism of action of NO were well appreciated before the discovery in 1980, when Furchgott and Zawadski reported that endothelial cells, when stimulated by acetylcholine, produced a vasodilator substance which relaxed vascular smooth muscle (5). A few years later, this endothelium-derived relaxing factor was identified as nitric oxide (6-8). This discovery revealed that mammalian cells were able to synthesize a free radical, NO, which could act both as a physiological messenger and as a cytotoxic agent. These observations marked the beginning of a new field of research, nitric oxide investigation, which has vastly expanded ever since (9). In 1992, the Science magazine named NO as the molecule of the year (10), and in 1998, the investigators responsible for the basic discoveries were awarded the Nobel Prize (11).

The importance of NO in the field of neonatology has become obvious during the past years (12). The vital role of endogenous nitric oxide in the early adaptation of pulmonary circulation has been shown (13). This has led to new strategies in the treatment of neonatal respiratory failure (14). Inhaled nitric oxide (iNO) has been shown to decrease vascular resistance and to improve oxygenation in persistent pulmonary hypertension of the newborn (PPHN) (15). Since the range of biological activities of NO is wide, it is

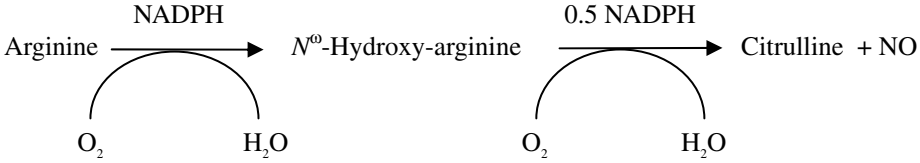
possible that not all of the major impacts have even been elucidated yet. NO reacts with a multitude of biological molecules, and awareness of possible toxicity must prevail.

This thesis was designed to increase our knowledge of pulmonary nitric oxide metabolism in the lungs and airways of term and preterm newborn infants. Both endogenous and exogenous NO was studied, to better understand possible side effects and therapeutic implications.

2 Review of the literature

2.1 Biosynthesis of nitric oxide

Most mammalian cells have a potential of generating nitric oxide (16). Enzyme nitric oxide synthase (NOS) catalyses five-electron oxidation of the terminal guanidino nitrogen atoms of L-arginine to generate L-citrulline and NO (17).



This reaction utilizes dioxygen and nicotinamide-adenine dinucleotide phosphate (NADPH) as co-substrates and tetrahydropteridin, flavin adenine dinucleotide, flavin mononucleotide, thiol and heme as co-factors (18).

The molecular simplicity of NO is in contrast to the complexity of NO synthase. NOS exhibits a bidomain structure homologous to cytochrome P450 reductase (19). It has an N-terminal oxygenase domain linked by a calmodulin recognition site to a C-terminal reductase domain. In its active form, NOS forms a tetramer where two NOS monomers associate with two calmodulins (18). The N-terminal domain of NOS contains binding sites for haem (*i.e.* iron protoporfyrin IX), tetrahydropteridin and L-arginine, and the C-terminal domain for flavin adenine dinucleotide, flavin mononucleotide and NADPH. Remarkably, each subunit attaches to at least five other molecules besides the three co-substrates and the partner monomer. Thus, 17 binding reactions are required to assemble a 300 kDa engine to generate a 30 Da product (20).

Three different isoforms of NOS have been identified. Three distinct genes for human neuronal (NOS1), inducible (NOS2) and endothelial (NOS3) NO synthase exist (21). The first isoform, NOS1, was purified from neurons of rat cerebellum (22). The inducible form of NOS was first isolated from mouse macrophages (23). Finally, NOS3 was first

identified in bovine aortic endothelial cells (24). The genes for human NOS isoforms have been located to the chromosomes 12, 17 and 7, respectively (18). Information of the enzyme structures at all levels from the primary amino acid sequence to the quaternary structure is available today (18).

NO synthesis may be regulated by either physiologic or pharmacologic agents (16). The availability and metabolism of L-arginine may be rate-limiting for NO synthesis (25, 26). Nitric oxide suppresses its own synthesis by binding to the haem iron moiety of NOS (27). Due to the stability of ferrous-nitrosyl complexes, any enzyme that forms a reduced ferrous-haem intermediate has the potential to be modulated by NO (28). NOS is no exception.

NOS1 and NOS3 are constitutively expressed under normal conditions and may produce picomolar levels of NO over several minutes (1, 21, 29). The enzyme activities are controlled by post-translational modifications and dependent on the intracellular Ca^{2+} concentration: NOS1 and NOS3 only bind calmodulin when Ca^{2+} is elevated (30). Acetylcholine, bradykinin and other agents that increase intracellular Ca^{2+} act as such agonists. Proinflammatory cytokines down-regulated the gene expression and activity of constitutive NOS in porcine pulmonary artery endothelial cells (31).

Phosphorylation through specific protein kinases is another post-translational regulation mechanism of NOS (32). Both its activity and subcellular distribution are affected by the degree of phosphorylation (30). In cultured porcine endothelial cells, fluid shear stress elicited the phosphorylation of NOS3, increasing its activity Ca^{2+}-independently (33). In contrast, the phosphorylation of NOS1 decreased its activity in rat brain neuronal cells (34). A non-phosphorylated, catalytically active NOS is bound to plasma membrane, generating NO that is released extra-cellularly (30).

NOS2, the high-output enzyme producing nanomolar levels of NO, is independent of elevated Ca^{2+} (1, 21). Ca^{2+}- independence is due to the exceptionally tight binding of NOS2 to calmodulin (35). Continuous synthesis of NO by NOS2 has also been demonstrated in normal human airway epithelium, suggesting that these differentiations are not straightforward (36). However, once induced, NO release may continue for days: NOS2 in mouse macrophages produced NO for as long as five days when inductive stimuli and the L-arginine substrate remained available (37). At the level of transcription, the activity of NOS2 is increased by cytokines, particularly by interferon-γ •••••γ•• interleukin (••) -1β , tumour necrosis factor-α (TNF-α) and bacterial lipopolysaccharide (21). A combination of these agonists was required for maximally activated NO synthesis in human alveolar and bronchial epithelial cells (38). Dexamethasone, epidermal growth factor, transforming growth factor-β, platelet-derived growth factor, insulin-like growth factor-1 and thrombin suppressed the cytokine-induced activity of NOS2 (21, 38-40).

Pharmacological regulatory agents include promoters and antagonists of NO production (16). The first group consists of organic nitrates that mimic NOS, *i.e.* NO donors, such as nitroglycerin (41). The antagonists include five classes of inhibitors of NOS actions (substrate analogs, binders of flavoprotein, calmodulin or haem, and depleter of tetrahydropteridin) and the inhibitors of induction of NOS2 (16).

2.2 Nitric oxide as a free radical

In biological systems, NO serves as an important intra- and intercellular messenger (42). As a free radical, it is oxidized, reduced or complexed with other biomolecules, depending on the microenvironment (43).

2.2.1 Reactivity of nitric oxide

Nitric oxide is an uncharged molecule composed of seven electrons from nitrogen and eight electrons from oxygen (44). This combination results in the presence of an unpaired electron, which makes NO paramagnetic and a radical (41). The majority of biological molecules contain bonds filled with two electrons. NO only reacts rapidly with the select range of molecules that have unpaired electrons in their outer orbital. They are typically other free radicals or transition metals, such as haem iron (44).

Nitrogen oxides of biologic relevance include elemental nitrogen in five oxidation states (NO_x: N_2O, $NO^{\cdot}$, $NO_2^{\cdot}$, NO_2^{-}, NO_3^{-}) (43). NO is one of the biologically active nitrogen oxides. Therefore, NO does not remain as $NO^{\cdot}$ -radical moiety in biological environment. In aqueous systems and at air-liquid interfaces, $NO^{\cdot}$ -generation yields nitrite (NO_2^{-}) and nitrate (NO_3^{-}) as end products (45). NO generates a chemiluminescent product upon reaction with ozone (41). The $NO^{\cdot}$ -radical reacts rapidly with the superoxide radical, forming highly reactive peroxynitrite anion ($ONOO^{-}$) (46). The broader chemistry of NO involves an array of interrelated redox forms implicated in the biochemistry of dioxygen: nitrosonium cation (NO^{+}) and nitroxyl anion (NO^{-}) (45).

In aqueous solution, the calculated half-life of NO at the nanomolar concentrations required for signal transduction would be more than 40 minutes (47). This is clearly longer than the biological half-life of NO, estimated to be close to five seconds (48). Even during this time, the lipophilic nature and small size of NO enable diffusion over several cell diameters and enable it to function as a transcellular messenger (49). NO may diffuse in and out of a cell membrane within a millisecond (44).

Nitric oxide forms complexes with transition metal ions, including those regularly found in metalloproteins (50). The main trap for NO is oxyhemoglobin, which binds NO faster by five to six orders of magnitude than oxygen (45). The reaction with haemoglobin produces nitrate and methaemoglobin (met-Hb) (51). The basis of many biological actions of NO is the activation of guanylyl cyclase through binding to the haem prosthetic group of the enzyme (52). Guanylyl cyclase increases the production of cGMP, modulating endothelium-dependent relaxation (53), platelet function (54) and nitrergic inhibitory transmission (55). Other NO-sensitive metalloproteins are NOS, cytochrome P450 (22), ferritin, ceruloplasmin, myoglobin, cyclo-oxygenase, catalase, ribonucleotide reductase and several components of the mitochondrial respiratory chain (56). These reactions have wide implications for the physiologic and toxic effects of NO.

Low molecular thiols, such as glutathione or cysteine, are likely to control NO homeostasis (57). NO reacts with sulfhydryl groups of thiols to form nitroso-thiols, possibly serving both to stabilize and to activate NO (50, 58). By slowly releasing NO, nitroso-thiols may form a protected way to effectively deliver an additional source of NO.

2.2.2 Toxicity associated with nitric oxide

Nitric oxide was previously known as a pollutant and a noxious gas present in the exhaust fumes from cars, which causes acid rain and destroys the ozone layer (59). The potential toxicity of endogenous NO still warrants consideration.

The direct toxicity of nitric oxide is modest. In activated macrophages, NO serves as an effector molecule, degrading the iron-sulphur centres, which results in the release of iron ions and iron-nitrosyl complexes (60). The same pattern of cytotoxity was found in hepatoma cells (60). *In vitro*, NO deaminated deoxynucleosides, deoxynucleotides and intact DNA at physiological pH, inducing mutations in the bacterial genome (61). Chronic intermittent (62) or continuous (63) exposure of animals to 0.5 – 2 parts per million (ppm) NO caused apparently nitrogen dioxide (NO_2) -independent degeneration of interstitial cells, interstitial matrix and connective tissue and emphysematous changes with large airspaces and destruction of alveolar septa.

The toxicity of NO increases greatly upon reaction with superoxide radical (O_2^-) (64). In both gas phase and aqueous solution, the rapid reaction with superoxide forms highly reactive peroxynitrite anion (46). At neutral pH, peroxynitrite forms peroxynitrous acid (ONOOH) (64). Although peroxynitrite has important microbicidal and tumoricidal functions, the generation of excess $ONOO^-$ leads to oxidative injury and lung damage (65). Specifically, peroxynitrite nitrates phenolic residues of tyrosines, forming nitrotyrosine, a marker of the toxic NO pathway (44). The detection of nitrotyrosine illustrates the site of peroxynitrite production and oxidative stress, providing evidence of the toxicity of NO in a number of diseases (66).

Nitrotyrosine has been detected from lung sections of patients and animals with acute lung injury (67, 68), idiopathic pulmonary fibrosis (69) and acute respiratory distress syndrome (ARDS) (70). Peroxynitrite degrades surfactant by causing nitration of the tyrosine residues of surfactant proteins, formation of lipid peroxides and loss of surface activity (71). Plasma nitrotyrosine was elevated in premature infants who developed chronic lung disease (CLD) (72). The presence of superoxide, transient metal, high concentrations of NO and oxygen and the absence of thiol groups, urate and ascorbate in the airways promote the destructive role of NO, as the generation of $ONOO^-$ is accelerated or the defence mechanisms against $ONOO^-$ toxicity are weakened (47, 73).

Reaction of peroxynitrous acid with target molecules may result in products characteristic of both NO_2 and hydroxyl radical (OH^-) as reactive intermediates (74). Furthermore, in the presence of oxygen, NO is rapidly oxidized to NO_2, which is a major pollutant. Even at very low concentrations NO_2 may acutely injure the distal airways and alveoli and disrupt the vascular endothelium (75, 76). However, as detected by chemiluminescence, the levels of NO_2 generated during clinical use of <40 ppm iNO remained below 0.3 ppm and were mostly undetectable (77).

Reaction of NO with oxyhaemoglobin yields methaemoglobin, an inactive oxygen transporter, which decreases the oxygen-carrying capacity of haemoglobin (78). The balance of met-Hb depends on its rate of production and the rate of elimination by methaemoglobin reductase in the erythrocyte (79). In a preterm lamb model, iNO at 80 ppm for 23 hours increased met-Hb up to 3.0%, which was low enough not to affect the oxygen-carrying capacity of blood (80). After lung transplantation in a Native American

Indian girl, ten hours of 80 ppm iNO transiently increased the met-Hb level up to 9% (81).

NO modulates platelet function by inhibiting platelet aggregation and adhesion (82). This may increase the bleeding time and the risk of intracranial haemorrhage or other bleeding disorders, especially in premature infants (83-85).

2.2.3 Antioxidant properties of nitric oxide

Being a free radical, nitric oxide has both pro- and antioxidant properties (47). NO can be protective against oxidative injury, depending on the specific conditions (86). A nitric oxide radical can both stimulate lipid oxidation and mediate oxidant-protective reactions in membranes (87). At high rates of NO production, the pro-oxidant *versus* antioxidant outcome depends critically on the relative concentrations of the individual reactive species (88). The pro-oxidant reactions of NO occur with superoxide, whereas the antioxidant effects of NO consequent to direct reactions with alkoyl and peroxyl radical intermediate during lipid peroxidation, terminating the propagation of lipid radical chain reactions (88).

NO limits injury to target molecules or tissues during events associated with excess production of reactive oxygen species. These include inhibition of oxidative killing of murine lung fibroblasts and mesencephalic neurons (89), attenuation of low-density lipoprotein oxidation (90, 91) and modulation (92) and reduction of ischemia-reperfusion injury (93).

Hydrogen peroxide mediates oxidation of different biological molecules that may result in tissue damage (89). NO does not react directly with $OH^{\cdot}$, but is able to protect cells against $OH^{\cdot}$ -mediated toxicity (56). NO induced ferritin, haem oxygenase, superoxide dismutase and endonuclease IV, which are protective proteins against oxidative stress, providing a cellular signal to up-regulate a variety of protective genes (94, 95).

In a premature lamb model of mild respiratory distress syndrome (RDS), 20 ppm iNO for five hours did not change significantly either the malondialdehyde and total antioxidant status levels in blood or malondialdehyde, reduced glutathione, glutathione peroxidase and glutathione reductase in lung parenchyma or amino-imino-propene bond generation, suggesting that short-term iNO did not increase oxidative stress and lung inflammation (96). In preterm rabbits, 14 ppm iNO for 20 hours decreased or prevented hyperoxia-induced oxidant stress and surfactant abnormality (97).

2.3 Endogenous nitric oxide in the lung and in the airways

Nitric oxide, a potentially toxic molecule, is formed endogenously in the human lung and is implicated in a wide range of biological functions (98). Lung cells capable of producing NO include macrophages, granulocytes, endothelium, fibroblasts, vascular smooth muscle cells, mast cells and epithelial cells, including type II alveolar cells (43). In the lung, both endogenous and exogenous NO reacts readily with a number of

molecules to form products with biochemical actions ranging from smooth muscle relaxation and vasodilatation (53), bronchodilatation (99), bacteriostasis through cytotoxicity (100), pulmonary capillary leak (101) and neurotransmission (55) to reactions with mutagenic potential (61).

2.3.1 Nitric oxide produced by inflammatory cells

The immunocytotoxic actions of nitric oxide require that NO should only be synthesized once an immune response has been triggered, and high levels of NO should be produced over a sustained period. The high levels of NO are synthesized by NOS2, which is not normally active, although it may be detected in some cell types (102, 103). However, when proinflammatory agents, such as endotoxin, TNF-α, INF-γ, IL-1 and IL-2 (104, 105), act on certain cells, they induce NOS2 activity (106). These microbial products and cytokines often act as synergistic pairs to stimulate the expression of NOS2 (20).

The activation of human NOS2 involves transcription of the messenger ribonucleic acid, which is triggered by the binding of some transcription factors, including nuclear factor-κB (107), to specific sites on the promoter of the NOS2 gene (106). The activation of newly synthesized NOS2 requires post-transcriptional alterations and translational and post-translational control (108).

In the immune system, NO production is one of the major antimicrobial mechanisms of macrophages (60). Sustained production of NO endows macrophages with cytostatic or cytotoxic actions against viruses, bacteria, fungi, protozoa, helminths and tumour cells (20). In a mouse cell line, viral replication was inhibited by INF-γ-induced NOS2 (109). NOS inhibitors have been shown to aggravate the course of various microbial diseases (110). Exogenous NO, in the form of NO-donating agents (111) or NO gas (112), hindered the growth of microbes. The survival of NOS2 knockout mice was markedly diminished upon infection compared to wild-type mice (113). Monocytes and activated macrophages also produce potent radicals, generating peroxynitrite, which decomposes into species with an extremely high oxidizing potential (64).

Inflammatory NO acts against tumour cells as well. Contrary to antimicrobial intracellular actions, this property requires extracellular killing. The specific mechanisms by which NO mediates tumoricidal effects remain to be elucidated. They may include combination of the suppression of protein synthesis and the inhibition of cellular respiration (114), impairment of DNA synthesis through the inhibition of ribonucleotide reductase (115) and direct toxicity through deamination reactions (116).

An example of a localized inflammatory disorder in the lung is asthma. Expired NO concentrations were higher in asthmatics than in normal subjects (117).

A generalized inflammation occurs in septicaemia, where the endothelium acts as the key organ. Endothelial activation is evident in septic patients, and the general endothelial dysfunction may cause the multiple-organ dysfunction (118). In a Finnish neonatal population, septicaemia caused 23% overall mortality and a very early-onset mortality rate of 30%, with group B Streptococcus (GBS) as the leading cause (52%) of the disease (119, 120). Nitric oxide plays an important role in the pathogenesis of septicaemia (121). In a lung model, marked NO synthesis occurred after exposure to exotoxin from *E. coli*

(122). In a series of 39 critically ill patients, septic patients had significantly higher plasma nitrate/nitrite level compared to trauma patients without infection (123). Duke *et al.* found higher serum nitrate/nitrite levels in septic children (median age 10 months) than in non-septic patients (124). Septic newborn infants, especially those with shock, had higher plasma nitrite and nitrate levels than healthy control newborns (125). There were no statistically significant differences between the plasma NO_2^- + NO_3^- levels of patients with gram-positive or gram-negative infections. A significant association between plasma NO_2^- + NO_3^- and TNF-α levels was noted.

2.3.2 Interaction of nitric oxide and the pulmonary surfactant system

Pulmonary surfactant is a multimolecular complex located at the air-water interface within the alveolus and synthesized by type II alveolar cells. It contains a surface-active lipid layer and an aqueous sub-phase, consisting of 80% phospholipids, 8% neutral lipids and 12% protein (126). From discrete sub-fractions, the strongly hydrophobic surfactant proteins (SP) B and C and the collectins SP-A and SP-D have been isolated. The main function of surfactant is to lower the surface tension of the air-liquid interface and to stabilize alveoli at low lung volumes. Hydrophobic proteins mainly improve surface activity, whereas collectins are predominantly involved in innate immunity (127, 128).

In a normal lung, the alveolar epithelial lining and the surfactant system are at least intermittently exposed to nitric oxide. NO has both indirect and direct effects on pulmonary surfactant (129). Inhaled NO has been reported to prevent endothelial damage and vascular leak in perfused lung (101, 130), decreasing the alveolar protein-rich exudate that inhibits surfactant function (97).

Directly, nitric oxide may either degrade or improve the pulmonary surfactant function (47). Peroxynitrite inhibited pulmonary surfactant function by lipid peroxidation and by damaging surfactant proteins (71). Inhaled NO and peroxynitrite, at concentrations likely to be encountered *in vivo,* decreased the ability of SP-A to aggregate lipids (131, 132). Surfactant dysfunction, caused by inhaled NO, was in part due to an alteration of the proteins in the epithelial lining fluid, which in turn, inactivated the surfactant (133). The mechanism of this dysfunction was further studied. Inhaled NO in haemorrhagic lung oedema and surfactant deficiency increased the inhibition of surface activity by converting haemoglobin to methaemoglobin in the alveolar exudate (134).

Alternatively, NO may have beneficial effects on surfactant function. Low-dose inhaled NO decreased surfactant aggregate conversion *in vitro,* protecting the alveolar surface (97, 134). It improved surface activity and also increased the large surfactant aggregates (135). In premature rabbits, a brief period of hyperoxia caused oxidant stress and decreased the surface activity of a surfactant, but low dosage of iNO decreased or prevented O_2-induced detrimental effects on the alveolar surface and alleviated oxidant stress (97). In a rabbit model of ARDS, combined surfactant therapy and iNO were more effective then either treatment alone (136).

2.3.3 Nitric oxide in the respiratory airways

Nitric oxide is synthesized in the upper and lower respiratory tract of adults, children, term newborn and preterm infants (12). The role of airway NO has been intensively studied, and exhaled NO in animals and humans has been repeatedly reported (137). During acute endotoxemia, exhaled NO levels reflected the serum NO concentrations (138) and appeared to originate from the alveolar compartment of the lung (139).

In humans, inflammation of the airways in adult asthma (117, 140) and bronchiectasis (141) increased the concentration of NO in exhaled air. Patients with ARDS had decreased NO concentrations in exhaled air compared to healthy controls (1.1 ± 0.4 *vs.* 5.5 ± 0.8 parts per billion [ppb], p = 0.0001 (142)). The exhaled NO levels of smokers were significantly lower than those of controls: the high NO concentrations in cigarette smoke may have damaged NO-producing cells and inhibited endogenous NO formation (140).

In healthy children aged 6 to 15 years, the basal endogenous concentration of NO in end-expiratory air was 8.7 ppb (143). In asthmatic children, exhaled NO levels were higher up to 31.3 ± 4.2 ppb (117). A similar increase has also been shown in acute wheeze in infants (144). Anti-inflammatory therapy decreased the exhaled NO levels in asthmatic children (145).

There are scarce data available on exhaled NO in infants. In healthy newborn infants, the peak nasal NO levels were 0.27 ± 0.01 ppm at the age of 1 hour, and a 30% increase was noted between 1 and 24 hours of age (146). In term infants, the same investigators found peak NO concentrations of 2.71 ± 0.44 ppm within 10 minutes after birth, which increased up to 3.81 ± 0.25 ppm at 4 to 7 days postnatally (147). In preterm infants, the peak NO values averaged 1.22 ± 0.16 ppm and the NO concentration increased significantly along with postconceptional age (147). Some previous measurements of the endotracheal tube gas of sick or anaesthetized infants have shown very low levels of NO [unpublished data (146)]. The NO excretion rate from the lower airways of three intubated preterm infants was 0.3 ± 0.5 nl/min (148).

NO-mediated airway smooth muscle relaxation has also been demonstrated throughout the newborn lung (149). Bronchoconstriction includes ventilation-perfusion inequality: the lung areas distal to the obstructed airways are perfused but not sufficiently ventilated. As nitric oxide relaxes both vascular and airway smooth muscle, it may serve as a mediator of the regional airflow - blood flow matching (99, 150). Inhaled NO produced bronchodilatation, reduced methacholine-induced bronchoconstriction (84), and improved ventilation-perfusion matching in human adults (151) and in animals (99, 152-154).

High levels of NO have been measured from human paranasal sinuses (155). Inhaled and exhaled NO was measured in adult volunteers and patients during spontaneous and controlled ventilation (156). In the nasopharynx of non-smoking volunteers, 20.3 nmol/min NO was synthesized, leading to autoinhalation of 0.07 to 0.13 ppm during inspiration. Smokers had reduced NO synthesis. 50 to 70 % of the inhaled NO was absorbed in the lung, while ventilated patients were deprived of NO autoinhalation. In adults, 40 to 45 % of exhaled NO originated from the intrathoracic airways, and intubation decreased exhaled NO by 54 to 59 % (157). The endogenous NO

concentrations of spontaneously breathing newborn infants were 12.0 ± 1.7 ppb during regular breathing and reached intermittent maxima of 52.2 ± 5.8 ppb (158). Autoinhalation of paranasally produced NO may regulate the ventilation-perfusion ratio in the lung (159) as well as the adaptation of the respiratory system to postnatal life (158).

2.4 Role of nitric oxide in the pulmonary circulation of the newborn

The inner layer of the vascular wall functions like an endocrine gland, as the endothelium is involved in the regulation of blood pressure and flow (160). Nitric oxide is one of the most potent substances released from the endothelium, and constitutive NO production is thought to maintain normal blood pressure (161). The importance of NO in the control of vascular integrity suggests that changes in NO homeostasis could play a role in the pathogenesis of human vascular disease (161). Both reduced and increased NO generation is associated with vascular pathology (162, 163).

Nitric oxide is a physiological vasodilatator synthesized in vascular endothelial cells (7-9). NO diffuses from the endothelium into adjacent vascular smooth muscle cells, where it activates guanylyl cyclase, catalysing the formation of cGMP (164). The intracellular accumulation of cGMP facilitates the phosphorylation of several proteins by the cGMP-dependent protein kinase, which leads indirectly to dephosphorylation of myosin light chains and thereby relaxation of smooth muscle cells (165). The NO that reaches the blood stream is rapidly inactivated by haemoglobin (166).

Endothelial NO synthesis is regulated by a variety of vasoactive agents, shear stress caused by the viscous drag of blood against the surface of the endothelium, and low arterial oxygen tension (167).

2.4.1 Foetal and transitional pulmonary circulation

The foetal pulmonary circulation consists of a high-resistance, low-flow circuit that accepts less than 10% of the combined ventricular output (168). Mechanisms contributing to the maintenance of high resting tone in the foetal lung are incompletely understood. They include vasoconstriction in response to low oxygen tension, lack of rhythmic distension, absence of an air-liquid interface and balance between the vasoconstricting and vasodilating mediators released by the endothelium (169). Endogenous NO activity is physiologically important in modulating the basal foetal pulmonary vascular tone (170). NO promotes angiogenesis, alveolarization and normal lung growth through the regulation of vascular endothelial growth factor: in NOS3-deficient mice, mild hypoxia impaired alveolarization (171). Vascular endothelial growth factor is also a potent vasodilator that mediates the release of NO (172).

At birth, as the lungs assume the function of gas exchange, pulmonary blood flow increases up to 10-fold, and pulmonary vascular resistance drops rapidly. By 24 hours of age, the mean pulmonary arterial pressure is approximately 50% of the systemic pressure. At the same time, pulmonary circulation undergoes dramatic structural adaptation, as the

endothelium is mechanically flattened or stretched within minutes of birth (169). Increases in oxygen tension, tidal ventilation, shear stress and release of vasoactive mediators play important roles in postnatal adaptation. Vasoactive mediators of major significance in the regulation of transitional vascular tone include NO, eicosanoids, endothelin and atrial natriuretic peptide (168, 173, 174).

Nitro-L-arginine, an inhibitor of NOS and NO production, attenuated markedly early adaptation, suggesting that increased release of endogenous NO is necessary for the smooth transition of pulmonary circulation at birth (13). In the foetal lamb, an increase in alveolar oxygen tension in the absence of foetal ventilation increased pulmonary blood flow by 8- to 10-fold, an effect that was blocked by nitro-L-arginine (175). Intrapulmonary infusion of nitro-L-arginine increased the pulmonary vascular tone of 0.78-term ovine lung (176). In isolated intrapulmonary arteries from late-gestation lambs, both basal and stimulated release of endothelial NO increased when oxygen tension was increased acutely (177). NO production was inhibited by acute and prolonged hypoxia in rat pulmonary arteries (178), suggesting that the increase in oxygen tension dilates the foetal pulmonary circulation *via* NO.

Parenchymal viscoelastic properties and airway calibre are also known to contribute to pulmonary resistance during the early postnatal period. Viscous resistance is the frictional resistance generated during the inflation or deflation of the lung. Inhaled and endogenous NO reduced the airway and tissue components of the resistance of the pulmonary airflow in the newborn piglet (149).

2.4.2 Ontogeny of pulmonary NOS isoforms

There are differences in the activities of the NOS isoforms during early pulmonary development. In normal mature lung, NOS1 was expressed in the airway epithelium and in non-adrenergic, non-cholinergic nerves, NOS2 in the airway epithelium and NOS3 in the vascular endothelium and the airway epithelium (160). In foetal rat lung, all three isoforms of NOS were expressed and present during lung development (179). In the epithelium, the cellular distribution of all the three isoforms was similar in foetal, newborn and adult sheep lung (180). The following changes in the expression of NOS isoforms during pre- and postnatal lung development have been found in experimental animals.

NOS1. The immunoreactivity of NOS1 was detectable in 13-day foetal rat lung (term = 22 days) (179). The expression of NOS1 protein increased 3.1-fold up to its maximal level at 20 days and decreased postnatally (181). In the foetal ovine lung, NOS1 was found in the epithelium at all levels of the respiratory tree, including the alveolar wall and airway and vascular smooth muscle (180).

NOS2. The immunoreactivity of NOS2 was detectable in 16-day foetal rat lung (179). In the foetal ovine lung, NOS2 expression was detected in the airway and vascular smooth muscle and in the bronchial and proximal bronchiolar epithelia, but not more distally (180).

NOS3. NOS3 protein has been demonstrated in the foetal ovine pulmonary vasculature as early as at 29% of term (182). Immunoreactivity of NOS3 was detectable in 14-day

foetal rat lung (179). The expression of NOS3 protein increased 3.8-fold up to its maximal level at 20 days and decreased postnatally (181). Similarly to NOS2, in the ovine epithelium, NOS3 expression was detected in the bronchial and proximal bronchiolar epithelia, but not more distally (180).

2.4.3 Failure of postnatal pulmonary adaptation

In some infants, the decrease of pulmonary vascular resistance does not take place shortly after the onset of air breathing, resulting in persistent pulmonary hypertension (183). This syndrome occurs in more than 1 per 1000 live births and results in substantial morbidity and mortality in otherwise normal infants (184). PPHN may be idiopathic or it may complicate a variety of neonatal disorders, including pneumonia, meconium aspiration, RDS (185), asphyxia, congenital heart disease, and pulmonary hypoplasia associated with congenital diaphragmatic hernia or oligohydramnios (186).

In PPHN, the abnormal gas exchange is largely due to excessive extra-pulmonary shunting. The intense pulmonary vasoconstriction causes blood to flow away from the lung *via* the foetal circulatory pathways, *ductus arteriosus*, foramen ovale or both. This results in arterial desaturation even in the presence of fully oxygenated pulmonary capillary blood. As arterial hypoxemia develops, venous hypoxemia becomes more profound, raising pulmonary vascular resistance independently of alveolar gas tensions, resulting in a vicious circle (187). Parenchymal lung disease, such as pneumonia, aspiration of meconium or amnion fluid and RDS, are commonly associated with PPHN.

The pathophysiology of PPHN includes complex alterations in the endogenous vasoactive mediator levels. Chronic NO inhibition during pregnancy caused pulmonary hypertension in newborn lambs (188). Infants with persistent pulmonary hypertension had low plasma concentrations of arginine and nitric oxide metabolites (189, 190). Circulating concentrations of the vasoconstrictive peptide, endothelin-1, were increased, and cGMP decreased in the plasma of infants with PPHN compared to healthy newborns (191). Among the eicosanoids and prostaglandins, prostacyclin appears to be a major vasoactive mediator in foetal lungs, although it may not be essential for a smooth postnatal transition (168).

2.5 Inhaled nitric oxide

Nitric oxide-releasing drugs, such as sodium nitroprusside and nitroglycerin, have been used for years to promote vasodilatation in the treatment of angina pectoris or as antihypertensive drugs (41). Nitrovasodilators are a chemically heterogeneous group of compounds, which release NO *in situ* and are used pharmaceutically as donors of NO (192).

Intra-venous vasodilatator drugs, such as tolazoline (193), prostacyclin (194) and sodium nitroprusside (195), may be effective in the treatment of PPHN, but their lack of specificity for pulmonary circulation limits their use for this indication (196). Inhaled NO improves the ventilation-perfusion mismatch by diffusing across the alveolar membranes

of the ventilated lung areas and by stimulating cGMP-mediated vasodilatation in the pulmonary vascular bed. Blood flow increases only in the well-ventilated areas of the lung, decreasing the ventilation-perfusion mismatch (197). Systemic hypotension does not develop, as NO is rapidly inactivated by haemoglobin.

2.5.1 Inhaled nitric oxide therapy in the newborn

Studies on term and near-term infants with respiratory failure have confirmed the beneficial effect of inhaled NO on oxygenation without concomitant deleterious side effects (198), which may include pulmonary toxicity, methaemoglobinemia and bleeding disorders.

A number of animal studies have provided information on the actions of inhaled nitric oxide in the lung. Exogenous NO, delivered by inhalation during mechanical ventilation, caused pulmonary vasodilatation in the mature lamb lung during pharmacologically induced pulmonary hypertension and acute hypoxia (199). In newborn porcine with meconium aspiration, 10 ppm iNO improved oxygenation and prevented the increase of cell apoptosis, but did not protect against early inflammatory damage caused by meconium aspiration (200). In the immature lamb, exogenous NO caused potent, sustained and selective pulmonary vasodilatation (201). In severe experimental RDS, 20 ppm iNO caused a marked and sustained improvement in pulmonary hemodynamics and gas exchange (202). It also increased pulmonary blood flow without worsening pulmonary oedema and decreased lung neutrophil accumulation (203). In an other experimental model, 20 ppm iNO did not increase oxidative stress or lung inflammation in premature lambs (96).

2.5.1.1 Term infants

In 1992, two study groups published series of infants treated with iNO. Roberts *et al.* gave 80 ppm to six critically ill infants for 30 minutes, and all of them showed rapid and significant improvement of preductal oxygen saturation (204). Kinsella *et al.* reported nine infants who had PPHN, were candidates for extra-corporeal membrane oxygenation (ECMO) and rapidly improved their gas exchange and arterial oxygen tension when treated with 10 - 20 ppm iNO (205). Two other prospective series have been reported. Bührer *et al.* treated ten consecutive newborns with severe hypoxemic respiratory failure with iNO doses ranging from 8 to 80 ppm (206). The optimal therapeutic concentration needed to improve oxygenation varied considerably between patients. Goldman *et al.* reported the following four patterns of response to iNO at 20 ppm for 20 minutes in 25 consecutive near-term infants with PPHN: 1. non-response; 2. acute transient response; 3. acute sustained response with successful weaning; 4. acute response and development of iNO dependence (207).

The first randomised, controlled trial was published in 1995 (208). 17 hypoxemic term/near-term neonates were randomly assigned to conventional treatment with or without adjunctive iNO up to 80 ppm. Inhaled NO produced a transient improvement in

oxygenation but did not reduce ECMO use. In an American multicentre study, the infants were •34 weeks of gestation and had an oxygenation index (OI) •25 (209). They received iNO 20 ppm with oxygen or oxygen alone. Altogether 114 iNO-treated infants and 121 controls were recruited. Significantly fewer iNO-treated patients had ECMO (39% *vs.* 54%, p = 0.014) and improved more their arterial partial pressure of oxygen (PaO_2; mean improvement 58.2 *vs.* 9.7 mmHg, p <0.001) and OI (a decrease of 14.1 *vs.* an increase of 0.8, p <0.001). No apparent effect on mortality was found. The same study group evaluated prospectively the effect of iNO for hypoxemia in PPHN (15). 58 full-term infants received either iNO 80 ppm or nitrogen as control gas. Inhaled NO doubled systemic oxygenation in 53% of the infants, whereas improvement was evident in only 7% of the controls. Inhaled NO reduced the requirement for ECMO (40% *vs.* 71%, p = 0.02). The same design produced similar results in a trial of 49 infants with PPHN (210): patients with a starting dose of 80 ppm showed improvement in PaO_2, oxygen saturation and OI when compared to baseline or controls, but no differences in mortality or ECMO use were seen.

To assess the dose-related effects of iNO, 155 term infants were enrolled in a placebo-controlled, double-masked trial (211). The treatments were 0 (control), 5, 20 or 80 ppm. With all the NO doses, the acute improvement in oxygenation was sustained, leading to a decrease in the requirement for ECMO. The effects of low-dose iNO were evaluated in a trial of 38 term or near-term infants with respiratory failure and PPHN (212). The initial iNO dose was 0 (control) or 2 ppm, which was increased up to 20 ppm if some deterioration occurred. At 2 ppm, iNO neither acutely improved oxygenation nor prevented clinical deterioration. However, iNO at 2 ppm attenuated clinical deterioration. At 20 ppm, iNO acutely improved oxygenation, but not in the infants previously treated at 2 ppm.

The different treatment strategies were studied in a trial of 205 neonates with severe PPHN (213). They were stratified according to diagnosis and randomly assigned to iNO and conventional ventilation or to high-frequency oscillatory ventilation (HFOV) without iNO. Treatment with HFOV and iNO was often more successful than treatment with HFOV or iNO alone. Specific diseases were associated with differences in responses.

A recent meta-analysis of iNO therapy for respiratory failure in term or near-term infants included 12 eligible randomised studies (214). Inhaled NO improved outcome by reducing the incidence of the combined endpoint of death and requirement for ECMO. The treatment significantly decreased the need for ECMO. However, mortality was not reduced. Oxygenation improved in approximately 50% of the infants receiving iNO. Within 30 to 60 minutes after commencing iNO, OI decreased by a weighted mean of 15.1, and PaO_2 increased by a mean of 53 mmHg.

2.5.1.2 Preterm infants

The iNO treatment of preterm infants has been evaluated in some prospective patient series (215) and in three randomised clinical trials, which have been combined into a meta-analysis (216).

Abman *et al.* published a case of a premature newborn with GBS sepsis, severe RDS and PPHN, who was successfully treated with iNO 20 ppm (217). In a series of eight premature infants (birth weights 520 to 1440 g, length of gestation 24 to 31 wk) with premature rupture of foetal membranes (PROM), oligohydramnios and pulmonary hypoplasia, iNO therapy improved significantly oxygenation and decreased mean airway pressure (MAP) (218). Altogether 50% of the patients died or developed severe intracranial haemorrhage. 23 premature infants with RDS were randomly assigned to receive 5 or 20 ppm iNO (219). Inhaled NO increased significantly arterial blood oxygen tension. No differences between the two NO concentrations was detected.

In an open, controlled trial, 42 infants <32 weeks of gestation were randomised into four treatment groups: 1. iNO 5 - 20 ppm for 72 h; 2. dexamethasone 0.5-1.0 mg/kg/day intravenously for 6 days; 3. both drugs together; 4. conventional treatment without either drug (220). There was no difference in the combined incidence of CLD and/or death before discharge between the groups. In the same study, changes in oxygenation and pulmonary hemodynamics were evaluated (221). Inhaled NO rapidly improved oxygenation and lowered pulmonary arterial pressure without affecting the long-term outcome.

Two other randomised controlled trials were published in 1999. In USA, 80 preterm infants (•34 weeks of gestation) with severe hypoxemic respiratory failure were randomly assigned to receive iNO 5 ppm or no iNO (222). In this double-blinded, multicentre study, iNO improved oxygenation after 60 minutes ($p = 0.03$). There were no significant differences in survival at discharge or in the incidence of CLD. No differences in serious side effects, namely intracranial haemorrhage or CLD, were seen, either. In a European multicentre study, altogether 85 preterm (<33 wk) and 119 near-term (•33 wk) infants with OI of 12.5 – 30.0 and 15.0 – 40.0, respectively, were recruited (223). The treatment was iNO 10 ppm, or control ventilation therapy without iNO. The decline in OI at two hours was greater in the iNO group than in the controls (median –6.2 *vs.* – 2.9, $p = 0.005$). The improvement in oxygenation was only significant in near-term neonates (median -7.8 *vs.* –2.7, $p = 0.03$). The survivors assigned to receive iNO spent fewer days on mechanical ventilation and in the neonatal intensive care unit. Again, the difference reached statistical significance only in the near-term infants. No differences in serious side effects (neurological complications, intracranial haemorrhage, cystic leucomalacia) were detected.

The results of the previous trials were combined into a meta-analysis (216). Among the111 infants receiving iNO, 44 deaths were observed compared to 40 deaths among the 99 control infants ($p = 0.91$). The Odds ratio (OR) was 0.97 (95% confidence interval [CI] 0.54 - 1.75). No significant difference in treatment failure, defined as death or chronic lung disease, was found (iNO 32/111 *vs.* controls 25/99; OR 0.77, CI 0.41-1.45). The rates of intracranial haemorrhage were similar (iNO 35/111 *vs.* controls 25/99; OR 1.37, CI 0.69 - 2.74).

The efficacy of iNO in chronic lung disease has been studied. In a case report, a 2-month-old and formerly 28-week premature infant with CLD and infected with respiratory syncytial virus was treated with iNO and HFOV after a failure of conventional therapy (224). The infant recovered without any need for ECMO. In another case, a 13-month-old infant with CLD and respiratory syncytial virus infection survived with iNO 20 to 80 ppm during seven days (225). 33 preterm infants with developing or

established CLD received 20 ppm iNO during seven days (226). Mean fraction of inspired oxygen (FiO_2) decreased from baseline 0.75 to 0.58 at 72 hours. No increase in the markers of oxidative or inflammatory injury was found.

2.5.2 Weaning from inhaled nitric oxide and long-term outcome

Dependency of nitric oxide inhalation, suggesting down-regulation of endogenous NO production, was reported in some earlier commentaries (227, 228). In a series of 40 children aged between 15 days to 17 years treated with iNO for respiratory failure, 11 patients showed decreased oxygenation after sudden discontinuation of iNO (229). The weaning strategies of iNO therapy were evaluated in a randomised, placebo-controlled, double-masked, dose-response clinical trial of 155 term infants with PPHN (230). It showed that withdrawal of iNO is not problematic if the initial response is successful, OI <10, and the iNO dose is gradually reduced to 1 ppm before cessation. The previous recommendation of transiently increasing FiO_2 at the cessation of iNO (231) was repeated.

The long-term outcome of infants treated with inhaled NO has been evaluated. One- and 2-year follow-up of a cohort of 52 infants with PPHN and treated with iNO showed 11.8% (1 year) and 12.1% (2-year) rates of severe neurodevelopmental disability (232). Mortality and neurodevelopmental outcome were assessed in a cohort of 24 very low birth weight neonates (<1500 g) who were rescued with iNO from severe hypoxemic respiratory failure (233). Although iNO acutely increased oxygenation, the survivors had high mortality, a high incidence of intracranial haemorrhage and a poor neurodevelopmental outcome. Another study described the long-term survival and neurodevelopmental status of 25 high-risk preterm infants enrolled into a randomised controlled trial of iNO therapy (234). Between the iNO-treated and control infants, no significant differences were found in mortality (12/20 *vs.* 8/22, OR 1.65, CI 0.87 - 3.3), neurodevelopmental delay (4/7 *vs.* 9/14, OR 0.89, CI 0.37 - 1.75), severe neurodisability (0/7 *vs.* 5/14, p = 0.12) or cerebral palsy (0/7 *vs.* 2/14, p = 0.53).

3 Purpose of the study

The ultimate clinical goal of research on nitric oxide during the neonatal period is to improve the results of neonatal respiratory care. The indications for iNO may be expanded, and both side effects and endogenous toxicity may be minimised. The purpose of this study was to evaluate endogenous NO metabolism in the lungs of preterm and full-term infants with severe respiratory failure, and to evaluate the efficacy and safety of iNO in a phase 1 trial. The specific aims were:

1. to investigate the evidence of inflammatory NO production in the lungs of infants with the fulminant form of early-onset pneumonia;
2. to evaluate the expression of nitrotyrosine and NOS isoforms in the lungs of infants with fatal respiratory failure, and whether inhaled NO therapy causes detectable immunohistochemical changes in nitrotyrosine or NOS isoforms;
3. to establish a new method for measuring the concentrations of exhaled and nasal NO from newborn infants intubated and ventilated for treatment of respiratory insufficiency;
4. to test the hypothesis that inhaled NO may be exceptionally effective in a select group of small premature infants who were likely to die soon after birth.

4 Subjects and methods

The ethics committee of the University Hospital of Oulu, Oulu, Finland, approved the protocols. The parents gave informed written consent. The number of patients included in the four series is shown in Table 1.

Table 1. Summary of the study patients and subjects.

Series	Cause of respiratory failure	Term infants	Preterm infants	Controls	Total
I Autopsied subjects					36
	Pneumonia < 4d	4	8	10	
	Pneumonia > 4d	1	4	9	
Patients					21
	Pneumonia, early-onset		7	7	
	IUI, no sepsis	1	6		
II Autopsied subjects					29
iNO-treated:	Chronic lung disease		4	4	
	Congenital heart defect	6		5	
	PPHN			1	
	Pulmonary hypoplasia	1		1	
	Bronchopneumonia	1		1	
Healthy lungs:				5	
III Exhaled NO					57
Patients reported:	RDS	2	6		
	Meconium aspiration	3			
	Tetralogy of Fallot	1			
IV Intractable respiratory failure					19
No contraindications:	RDS and sepsis		3	2	
	RDS and pneumonia		2		
	RDS			1	
	Pulmonary hypoplasia			1	

4.1 Subjects and patients

Series I. The documents of the autopsies performed on infants in the Department of Pathology, University of Oulu, during the years 1986 - 1998 were reviewed. A paediatric pathologist reconfirmed all the autopsy diagnoses from histological lung specimens. Twelve infants with the following inclusion criteria for lethal early-onset bacterial pneumonia were identified: onset of disease <72 h age and histological finding of pneumonia associated with sepsis proven by blood cultures or by compatible clinical course (shock, hypoxemia, progressive course, abnormal white blood cell count). According to the pathologist's report, pneumonia/sepsis either caused (n = 8) or contributed (n = 4) to death. The following three groups were additionally studied: death at an age of less than four days without infection, infants dying of pneumonia and sepsis with onset later than 72 hours of age, and infants older than four days dying without infection.

Altogether 21 newborns requiring mechanical ventilation were studied. Seven infants suffered from fulminant early-onset pneumonia; in all cases the mother had had ascending intra-uterine infection (IUI). Control infants of similar gestation had no symptomatic infection at birth, despite maternal IUI. The third group of infants had neither neonatal infection nor chorionamnionitis.

Series II. Among the autopsies reviewed in the Department of Pathology, University of Oulu (series I), and in the Children's Hospital, University of Helsinki, during the years 1985 - 1999, altogether twelve infants had received inhaled NO therapy before death (iNO group). For each of them, a paired control was found and matched for diagnosis, gestational age at birth, age at death and the need for extra-corporeal perfusion during cardiac surgery. The autopsy diagnoses in the iNO group were CLD, congenital heart defect requiring cardiac surgery, pulmonary hypoplasia and bronchopneumonia. In the iNO group, the congenital cardiac malformations were agenesis of the right coronary artery with cardiac hypertrophy and fetal hydrops (case 5), tetralogy of Fallot (case 6), total anomalous pulmonary venous drainage with atrioventricular septal defect and double-outlet right ventricle (case 9) and hypoplastic left heart syndrome (cases 10, 11 and 12). The controls had double-outlet right ventricle with fetal hydrops (control 5), coarctation of aorta (control 6), hypoplastic left heart syndrome (control 10), truncus arteriosus communis and ventricular septal defect (control 11) and congenital aortic stenosis (control 12). Pulmonary hypoplasia in the iNO case 8 was idiopathic, whereas control 8 had diaphragmatic hernia. The iNO case 7 and control 7 had bronchopneumonia. Two subjects had tracheitis in bronchoscopy (iNO case 11 and control 11). Two subjects showed clinical symptoms of septicaemia (iNO case 12 and control 12). Three subjects had PPHN (iNO case 4 and controls 4 and 9), and three had secondary pulmonary hypertension (iNO cases 7, 8 and 9).

An additional group of five infants were studied. These infants did not have severe respiratory failure prior to death. They were compared to the subgroup of infants with severe respiratory failure, similar gestation at birth and similar postnatal age (pairs 4, 7, 9, and 10).

Series III. Altogether 57 infants were evaluated for the study of developing a method for exhaled NO measurements. The infants suffered from respiratory failure and were

nasotracheally intubated and ventilated using a pressure-controlled ventilator with a patient-triggered ventilation device (Infant Star with StarSync, Nellcor Puritan Bennett Inc., Pleasanton, CA). When all the details of the method had been refined, the results of the measurements from prospectively recruited six preterm and six full-term infants were reported.

Series IV. For very early iNO therapy, the eligible patients had birth weight <1500 g and progressive respiratory failure before seven hours' of age. Respiratory failure was defined as a failure to attain adequate arterial oxygen tension on two consecutive determinations, despite 100% oxygen ventilation, using a mean airway pressure of at least 13 cmH_2O and early surfactant therapy (Curosurf®100 mg/kg). For an assessment of the degree of respiratory failure, OI values and arterial-alveolar ratios for oxygen tension (a/A-ratio) were calculated. Cardiac Doppler ultrasound examination was performed to confirm supra-systemic arterial pressures prior to iNO. The contraindications were lethal malformation, unevacuated pneumothorax, extreme prematurity (gestation < 24+0 weeks), severe shock (blood pressure below -2 SD of the mean despite treatment) and severe bleeding disorder with disseminated coagulopathy judged from the low platelet count.

Patients were recruited between May 1997 and December 2001. Altogether 246 very-low birth weight infants were admitted into the neonatal intensive care unit of Oulu University Hospital, and 19 of them fulfilled the eligibility criteria. Nine of them had no contraindications. Four were not treated with iNO. In three instances the research team was not informed in time, and in one case, there was a technical failure in the NO administration device.

The initial dosage of iNO was 20 ppm, and after an adequate response, it was titrated to the lowest level that maintained oxygenation. Weaning was performed gradually. NO and NO_2 levels from the expiratory limb were monitored continuously, and blood methaemoglobin was measured every 12 hours. Airway specimens were collected during routine suctioning and analysed for inflammatory mediators.

4.2 Methods

4.2.1 Immunohistochemistry and analysis of autopsy specimens

The lungs were fixed in formalin, and paraffin blocks were made. For immunohistochemistry (235), five-micron sections of the blocks were mounted on Super Frost Plus microscopic slides and incubated overnight at room temperature. The specimens were deparaffinized and rehydrated. Treatment of the tissue sections in hydrogen peroxide for 15 minutes quenched the endogenous peroxidase. Individual slices were immunostained with monoclonal NOS1, NOS2 and NOS3 antibodies (4 µg/ml; Transduction Laboratories, Lexington, KY) and with polyclonal nitrotyrosine antibody (1:750; Upstate Biotechnology Inc., Lake Placid, NY) or buffers (negative control). The specimens were incubated at room temperature overnight. After the addition of

biotinylated secondary antibody, streptavidin-peroxidase was bound to the secondary antibody, and the complex was stained with chromogen for a red signal. The sections were counterstained with hematoxylin. Both the addition of dithionite and the coincubation of nitrotyrosine together with the antibody completely blocked the immunoreactivity, indicating that the antibody specifically reacted with the nitrotyrosine moieties. The specificity of NOS2 antibody was verified by analysing the lysate of lipopolysaccharide-stimulated and control macrophages using Western blots. Stimulated macrophages revealed an immunoactive protein of about 80 kD, whereas the control showed no immunoreactivity.

In series I, the degree of immunostaining for NOS2 was regarded as acceptable when there was positive staining for NOS2 in bronchial epithelial cells: NOS2 appears to be constitutional in bronchioles (236). The absence of NOS2 staining or nitrotyrosine staining in lung cells was confirmed by processing another specimen.

The immunostaining of the different cellular compartments in the lung was investigated. In series I, the following cell types were individually evaluated for immunostaining: pulmonary alveolar macrophages (AM), interstitial macrophages, neutrophils, bronchial epithelial cells, alveolar epithelial cells, vascular endothelial and smooth muscle cells, interstitial fibroblasts and mucous glandular epithelium. Alveolar exudate was additionally graded for nitrotyrosine. In series II, NOS and nitrotyrosine immunostaining was examined along the respiratory tree from the bronchial to the alveolar level. The epithelia of bronchi, bronchioli, terminal and respiratory bronchioli, alveolar ducts and alveoli were evaluated. The immunostaining of the vascular endothelium adjacent to the proximal and distal airways was studied. The immunostaining of pulmonary AM, bronchial and bronchiolar smooth muscle as well as pulmonary vascular smooth muscle was evaluated. In addition, the alveolar lining and any non-cellular material present in the alveolar spaces were studied on the basis of nitrotyrosine immunoreactivity.

The immunostaining of cells was graded by the intensity of the colour deposit within the cells and/or by the number of cells stained as follows: 0 = negative (no immunostaining in any cell), 1 = faint (positivity in < 25% of cells and/or faint colour), 2 = faint to moderate (positivity in > 25% of cells and/or clear colour), and 3 = moderate to strong (positive staining in all cells and moderate to strong intensity of colour). To avoid the bias of a subjective interpretation of the immunohistochemistry findings, two investigators (K.V., O.A.) blindly and independently evaluated the study samples.

4.2.2 Analysis of airway specimens

Airway specimens from intubated infants were collected at 4 to 6 hours' intervals during the first week of life according to the nursery protocol (237). Immediately after recovery, a fraction was frozen at -80°C and used for the analysis of cytokines and SP-A. Another fraction was centrifuged at 150 x g for 10 min. The pellet containing the cells was frozen at -80°C and subsequently used for Western blot analysis of NOS2 and nitrotyrosine (238). Prior to the analyses, 3 to 6 successively recovered specimens were combined. The specimens were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

The gel-loading artefact was corrected on the basis of DNA. Proteins were transferred on to a nitrocellulose membrane. Thereafter, the membranes were incubated with either NOS2 or nitrotyrosine antibody, and the specific antibody staining was visualized using peroxidase-conjugated secondary antibody and quantitated by video imaging and densitometry. IL-1β, IL-8 and TNF-α were quantitated using ELISA (R&D Systems, Minneapolis, MN). The methods were validated for the analysis of airway specimens. SP-A was quantitated using a SP-A kit TDR-30 (Teijin Ltd., Japan). The mean results of the airway specimens collected during 48 h are reported. The cytokine and SP-A concentrations were expressed on the basis of both the volume of the airway specimen and the volume of the epithelial lining fluid. The latter was calculated using the urea method (237).

4.2.3 Exhaled nitric oxide measurements

A rapid-response chemiluminescence analyser (NOA 280, Sievers Instruments, Boulder, CO) was used. NO gas reacts with ozone, producing energy in the form of light that is proportional to the amount of NO and can be measured using a luminometer (239). The analyser sample flow rate was configured to 100 ml/min. The manufacturer-specified lower limit of sensitivity for the NO analyser was <1 ppb and the response time for 90% full scale <0.2 s. The measured response time was 0.1 s. A square wave pulse of NO with a duration of 0.17 s or more could be accurately determined with a more than 90% response. During the measurements, the shortest expiratory time recorded was 0.29 s and the mean expiratory time was 0.87 ± 0.09 s.

Calibrations. First, the sample tube was tightly connected to a Zero Gas Filter (Sievers Instruments, Boulder, CO), and room air was vacuumed through the device for five minutes. The linearity of the analyser response was verified by four repeated calibrations, which showed a lower limit of <1 ppb for our instrument. Secondly, the sample tube was connected to a gas bottle containing 80 or 100 ppm of NO in nitrogen (Oy AGA Ab, Espoo, Finland), which served as a standard test gas. NO was vacuumed for 5 min, and the test gas calibrations were performed four times as well. When the Breath program is used, the sampling system causes an accurately defined pause between the pressure recording and the NO signals. To synchronize the signals, NO/pressure-offset calibration was performed by injecting 5 ml of the test gas into the combined sample and the pressure tube four times.

Ambient NO. Ambient NO was recorded as previously recommended (240, 241). Before and after each session, the NO concentrations of ambient and incubator air were measured. Similarly, the NO concentrations of hospital compressed air were measured through a flow meter (Bird Products, Palm Springs, CA). The ambient NO concentration did not associate with the peak expiratory NO levels (data not shown), allowing unbiased NO measurements (242).

Lung model. The validity of the clinical measurements was ascertained using a lung model, which was composed of inert materials. Similarly to the infant's lung, the test lung was connected to the respirator and NO analyser *via* an intubation tube. Between the intubation tube and the respirator circuit, a size-fitted adapter, normally used for

surfactant administration (RSP Respiratory Support Products Inc., USA), was placed. The gas sample tube was connected to the side port of the adapter without any increase in the ventilatory dead space.

During the testing, the respirator settings were within the therapeutic range. The test lung was filled with dry room air containing a known concentration of NO. Starting with expiration, the test lung was mechanically ventilated, and the concentration of NO was continuously measured. Sharp NO peaks were evident. The maximal NO concentration recorded during the first expiration accurately reflected the actual NO concentration. The response was linear over the range of 1 to 333 ppb, as determined with the test gas ($r = 0.99$, intercept 0.0). The response time of 0.1 s for a >90% response was verified.

The expiratory flow is likely to influence the mixing of the gases from the lung and the respiratory circuit, resulting in possible inaccuracy of the NO concentration at low expiratory flow rates. Therefore, the dependence of the measured NO concentrations as a function of the expiratory flow rate was studied using the following setting. A syringe was attached to an infusion pump and connected *via* the intubation tube to the respiratory circuit and the test lung. A given concentration of NO (333 ppb) was infused using different rates during mechanical ventilation. The peaks in NO concentration were not affected when the expiratory flow rate exceeded 1.5 ml/s. The result was obtained using a range of clinically applicable respirator settings. On the basis of this evidence, the analysis of NO values was limited to the breaths with expiratory flow rates exceeding 1.7 ml/s and duration of >0.2 s.

Expired NO measurements. All the measurements were made on a daily basis for as long as the infant required assisted ventilation or until the age of six days, whichever came first. During the measurement, the patient was lying in a supine position in a closed incubator. The temperature in the incubator was set at 31 to 35°C for preterm and at 29 to 31°C for term infants. The incubator humidity was 60 to 65% and the room temperature 23 to 25°C during the measurements. The inspired gas consisted of a mixture of hospital compressed air and oxygen. FiO_2 ranged from 0.21 to 1.0.

Each measurement consisted of four periods of 60 s. All measurements were performed with the Breath program, which displayed the NO concentration and the airway pressure curves. The maximal NO was the highest expiratory NO peak detected during the four measurement periods. In addition, the median value of all expiratory NO peaks was calculated.

Nasal NO measurements. Upper airway measurements were performed with the Nasal program, which displayed only the NO concentration curve. The smallest volume for which more than 90% of the actual NO concentration could be recorded was 0.17 ml.

The sample tube was disconnected from the adapter. For tight fitting, a naso-gastric flexible catheter of size 5 (Vygon, Ecouen, France) was cut at 2 mm from the coupler, which was connected to the sample tube. An assistant held the sample tube in the free nostril. One measurement consisted of four successive periods of 30 to 60 s. Maximal and baseline NO values were recorded. The maximal nasal NO peak value was the highest NO peak value detected during the four measurements.

Measurement of lung mechanics and analysis of NO values. The lung mechanics were measured using a spirometer (StarTrack, Nellcor Puritan Bennett Inc., Pleasanton, CA). It was connected to the respirator circuit through a flow sensor, which was calibrated and tested to be accurate at flow rates <15 l/min. There was a dead space of <1 ml. The

spirometer displayed tidal volumes and pressure and flow curves, while the NO analyser displayed NO and pressure curves.

The peak NO values, duration of expiration, flow rates, tidal volumes and minute ventilation were recorded. Each exhaled NO peak and the corresponding volume peak were identified one by one based on the similar shapes of the pressure curves. Respirator-induced and spontaneous breaths were analysed separately. They were differentiated using the ventilator setting-matched pressure and volume peaks. The recordings were analysed when the expiration had sufficient duration (>0.2 s) and flow rate (>1.7 ml/s). For the results, the hospital compressed air NO levels were subtracted from the exhaled NO concentrations. The expiratory NO peaks correlated with the following respiratory characteristics: tidal volume, airway pressure, flow rate, minute volume and severity of respiratory failure. For assessment of the degree of respiratory failure, the following indices were calculated: ventilatory index (VI = MAP x FiO_2 x 100), OI (OI = VI / preductal PaO_2, mmHg) and a/A-ratio [a/A = PaO_2 / (713 x FiO_2 – arterial carbon dioxide tension)]. The formula to convert mmHg to Pascals: 1 kPa = mmHg / 7.5.

4.2.4 Statistical methods

The statistical analysis was performed using the StatView (StatView II, 1987-88, Abacus Concepts Inc., Berkeley, CA) statistical software unless otherwise indicated. A p-value of <0.05 was considered statistically significant.

In series I, the results were expressed as means ± SE. The percentage distribution of the different grades of immunostaining in fulminant early-onset pneumonia was compared to that in age-matched controls and in pneumonia/sepsis of later onset using contingency table analysis. The correlation between NOS2 and nitrotyrosine immunostaining was analysed using Spearman's rank correlation test. The patient characteristics and the results of the airway specimen analysis were compared using analysis of variance or the chi-square test as appropriate.

In the series I and II, the consistency between the two investigators scoring the immunohistochemistry was analysed by means of the κ coefficient (243). This coefficient is different from the correlation coefficient and serves as an estimate of observer agreement on categorical data. The values of κ coefficients range between 0.0 and 1.0, with 0.0 meaning poor agreement and 1.0 perfect agreement. The mean κ coefficients for cellular staining ranged from 0.32 to 0.63 in series I, indicating fair to substantial agreement, and from 0.25 to 0.43 in series II, indicating fair to moderate agreement. The level of bias due to variability between the two observers was low enough for the results to be reliable in semi-quantitative analysis.

In series II, the results were expressed as medians and inter-quartile ranges, unless otherwise indicated. To avoid type I errors in final conclusions, the multiple comparisons were corrected by multiplying the statistically significant p-values by the number of pair wise comparisons being made. The distribution of scoring in the iNO group was compared to that in the paired controls using the Wilcoxon signed-rank test. Correlations between the degree of immunostaining of the different isoforms of NOS and nitrotyrosine and correlation between nitrotyrosine in the two different compartments were calculated

using Spearman's rank correlation test and the multiple regression test. The frequencies of immunopositive compartments were compared between the lungs of the infants who died of severe respiratory failure and those of the age-matched control infants. The differences in the frequencies were evaluated using Fisher's exact test.

As studied in four lungs processed separately for immunohistochemistry, the inter-assay variation coefficient of the immunohistochemical grades was 6 %. On the basis of this evaluation, it was concluded that the level of bias due to methodological variation was adequate for semi-quantitative analysis.

In series III, the results were expressed as median (range) or mean (± SE), as indicated. The statistical evaluation was performed using the SPSS for Windows 9.0 software (SPSS Inc., Chicago, IL). The differences between the two groups were compared with paired or unpaired *t*-test, as applicable. The correlation between the parameters was determined by a correlation coefficient.

5 Results

5.1 Nitric oxide synthases and inflammatory markers in early-onset neonatal pneumonia (I)

5.1.1 Autopsy specimens

The duration of gestation was 32 ± 2 wk in the early-onset pneumonia group, 29 ± 2 wk in the controls who died at an age of less than four days, 28 ± 3 wk in the group with pneumonia of later onset and 35 ± 2 wk in the older controls. The mean postnatal ages at death were 24 ± 10 h, 36 ± 11 h, 3 ± 2 months and 2 ± 1 months, respectively. In fatal pneumonia, moderate to massive quantities of polymorphonuclear leukocytes were detected in the lung interstitium and alveoli. All specimens contained alveolar and interstitial macrophages in variable quantities that did not correlate with the presence of infection. Hyaline membranes were found in six cases. Of the nine positive blood cultures in fulminant septicaemia, six were GBS, one viridans streptococcus, one pneumococcus and one *E. coli*. Among the late infections, two blood cultures were positive for Enterobacter, while one had Parainfluenza serotype 3 antigen and one respiratory syncytial virus antigen.

Of the twelve infants who died of fulminant early-onset pneumonia, eleven (92%) had no or faint NOS2 staining associated with AM (Figure 1). Four (80%) of the infants who died of late-onset pneumonia/sepsis showed strong NOS2 staining of alveolar macrophages. All control infants, regardless of their postnatal age, had NOS2-positive AM.

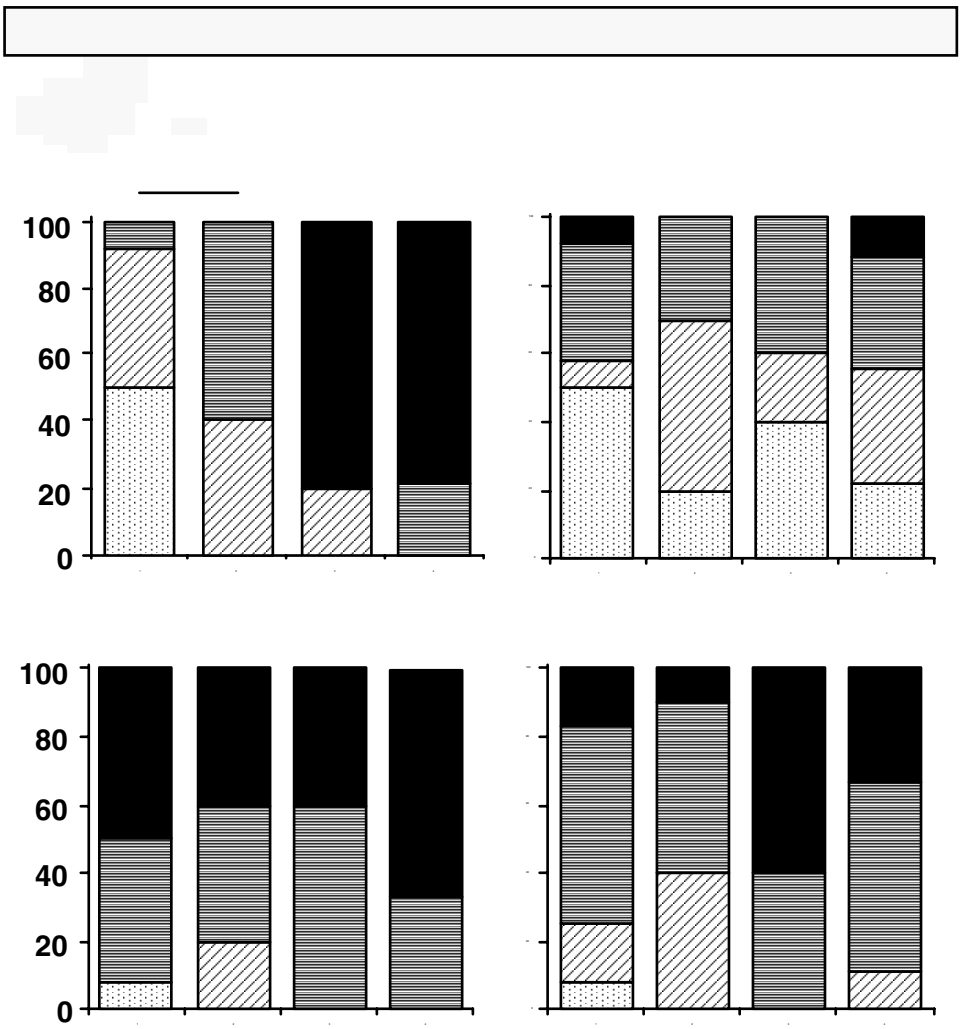


Fig. 1. NOS2 immunostaining of the lung cells from autopsy specimens. The percentage distributions of the scores are shown. * p < 0.05

Bronchial epithelium, mucous glandular epithelium and vascular smooth muscle cells revealed moderate to strong NOS2 immunostaining in 67 to 98% of cases, regardless of diagnosis or postnatal age. The alveolar epithelium and the vascular endothelium showed moderate to strong immunostaining for NOS2 in 36 to 42% of cases. Again, the presence of infection and postnatal age did not affect immunostaining. Immunostaining for NOS2 was weakly detectable in interstitial fibroblasts (2 cases) and neutrophils (3 cases).

Alveolar macrophages did not show strong nitrotyrosine immunostaining in any specimen. They showed moderate nitrotyrosine positivity in 8% of the early-onset pneumonias and in 40% of the pneumonias of later onset. AM showed no or weak nitrotyrosine positivity in 92% in the early-onset pneumonia group and in 60% in the group with pneumonia of later onset.

In alveolar exudate, moderate to strong nitrotyrosine positivity was seen in 16% of specimens in the early-onset pneumonia group and in 60% in the late-onset group ($p = 0.06$). There was no or weak nitrotyrosine positivity in the alveolar epithelium in 100% of specimens in both early- and late-onset pneumonia. Bronchial epithelium showed no or weak nitrotyrosine positivity in 75% of specimens in the early-onset pneumonia group and in 60% in late-onset pneumonia. In the interstitium, these proportions were 92% and 60%, respectively. Mucous glandular epithelium showed no or weak nitrotyrosine positivity in 92% of early-onset pneumonia and in 80% of late-onset pneumonia. In neutrophils, vascular endothelium and vascular smooth muscle cells, no significant nitrotyrosine positivity was detected in either group.

There was a single patient in the early-onset pneumonia group with moderate NOS2 expression in alveolar macrophages. He had no nitrotyrosine positivity in AM. However, taken together, NOS2 positivity correlated with nitrotyrosine immunoreactivity in AM ($r = 0.47$, $p = 0.005$) in bronchial epithelium ($r = 0.37$, $p = 0.03$) and in alveolar epithelium ($r = 0.33$, $p = 0.05$).

5.1.2 Airway specimens

Three groups of infants with similar birth weight and gestational age, requiring mechanical ventilation at birth, were studied. Of the infants with fulminant early-onset pneumonia, five had severe RDS and PPHN. Three had GBS sepsis, one *Klebsiella*, one *E. coli* sepsis and two negative blood cultures. The control infants had less severe respiratory disease than the infants with early-onset pneumonia.

The cell fractions from the airway specimens were analysed for NOS2 and nitrotyrosine. In early-onset pneumonia, the concentrations of both NOS2 and nitrotyrosine were lower than in the newborn exposed to IUI but with no detectable infection. In early-onset pneumonia, NOS2 and nitrotyrosine increased during the course of recovery ($p < 0.05$, paired t-test). Western blots showing NOS2 and nitrotyrosine immunoreactivity from a patient with early-onset pneumonia are shown in Figure 3 of series I. NOS2 and nitrotyrosine were low at birth, increased during the recovery phase and returned to low levels after recovery.

The concentrations of IL-1β, IL-8, TNF-α and SP-A in the airway specimens were analysed. TNF-α was low in each group. Infants with fulminant early-onset pneumonia and controls had lower IL-1β and SP-A than those without detectable infection despite IUI. The chemokine IL-8 was moderately increased in the first airway specimen already in the infants with early-onset pneumonia. In early-onset pneumonia, IL-1β, IL-8 and SP-A increased during recovery ($p < 0.05$, paired t-test). When expressed on the basis of epithelial lining fluid, the differences in IL-1β and SP-A between the three groups remained similar.

5.2 Pulmonary NO synthase and nitrotyrosine expression in respiratory failure and influence of inhaled NO (II)

Twelve infants received iNO as compassionate treatment for severe cardiorespiratory failure. There were no significant differences between the iNO-treated and control infants in gestational age at birth [39 (27, 41) wk *vs.* 39 (27, 40) wk], birth weights [2850 (892, 3988) g *vs.* 3425 (895, 3938) g] or postnatal age at death [15 (3, 66) days *vs.*13 (2, 150) days]. The median duration of continuous iNO treatment was 7 (1, 20) days, and the median maximal dose of iNO was 32 (18, 60) ppm. All infants received abundant supplemental oxygen and mechanical ventilation prior to death. No inhaled NO was given to the controls, either, because iNO therapy was not available or because it was not deemed indicated.

In fatal respiratory failure, accumulation of cellular debris or inflammatory cells, extravasation of erythrocytes into the alveolar space and desquamation of mucosa were equally evident in both groups' specimens. Hyaline membranes were present in two cases (iNO case 8 and control 8). There were no detectable differences in the number or distribution of inflammatory cells (AM, interstitial macrophages, granulocytes) between the iNO and control groups. Two infants (iNO case 7 and control 7) had viral pneumonia. Parainfluenza-3 antigen was positive in the serum of iNO case 7. All the blood cultures preceding death remained negative.

5.2.1 Immunohistochemistry of NOS1

In several cases, immunostaining of NOS1 was evident in the epithelium at all levels of the respiratory tree. Staining was most abundant in the proximal airways and faded towards the alveoli ($p = 0.0001$, Wilcoxon signed-rank test). The vascular endothelium in the vicinity of the respiratory tree was faintly to moderately immunopositive. Vascular endothelium as well as vascular and airway smooth muscle layers contained some NOS1 immunostaining that did not fade distally. Alveolar macrophages showed moderate to strong immunostaining for NOS1.

To find out whether iNO treatment changed the immunostaining of NOS, the results were compared between the two groups with respiratory failure. There were no detectable differences in NOS1 immunostaining between the iNO-treated specimens and the matched controls at any level of the airways and the alveoli. There were no differences in the staining of alveolar macrophages, either.

5.2.2 Immunohistochemistry of NOS2

Immunostaining of epithelial cells was positive for NOS2 at all levels of the respiratory tree, decreasing towards the distal parts ($p = 0.0001$, Wilcoxon signed-rank). Along the vascular endothelium, NOS2 immunostaining was detectable at all levels of the

respiratory tree. The airway and vascular smooth muscle showed some NOS2 staining. Alveolar macrophages were positive for NOS2.

Immunostaining of NOS2 in the bronchiolar epithelium was stronger in the iNO-treated group than in the control group ($p = 0.03$, Wilcoxon signed-rank). This result could have been obtained by chance ($p = 0.12$ after correction for multiple comparison). Immunostaining of NOS2 in the bronchial smooth muscle was stronger in the iNO group ($p = 0.04$, Wilcoxon signed-rank; $p = 0.16$ after correction for multiple comparison). There was a trend towards stronger NOS2 immunostaining in the bronchiolar smooth muscle in the iNO-treated group compared to the controls, but the differences did not reach statistical significance.

5.2.3 Immunohistochemistry of NOS3

The epithelium of the respiratory tree revealed NOS3 immunostaining that decreased towards the periphery ($p = 0.0002$, Wilcoxon signed-rank). The vascular endothelium around the airways was moderately to strongly immunostained. The airways and vascular smooth muscle were also immunopositive. Alveolar macrophages showed moderate to strong NOS3 immunostaining.

There were no significant differences between the iNO-treated and control patients regarding immunostaining of NOS3.

5.2.4 Immunohistochemistry of nitrotyrosine

Nitrotyrosine immunostaining was detected along the respiratory epithelium without differences between the proximal and distal parts of the respiratory tree ($p = 0.2$, Wilcoxon signed-rank). The vascular endothelium showed mainly negative staining. Also, the smooth muscle layers of the airways and blood vessels had little, if any, nitrotyrosine positivity. However, substantial quantities of nitrotyrosine immunostaining were found in alveolar macrophages. The alveolar exudate and hyaline membranes were also immunoreactive to nitrotyrosine.

There were no significant differences in nitrotyrosine immunostaining between the two groups in any of the cellular compartments studied. Nitrotyrosine immunoreactivity in the extracellular lining fluid correlated significantly with nitrotyrosine immunopositivity in alveolar macrophages ($r_s = 0.5$, $p = 0.03$) but not with nitrotyrosine positivity in the alveolar epithelium ($r_s = 0.4$, $p = 0.06$).

5.2.5 Association between alveolar nitrotyrosine and NOS

To clarify the association between the immunoreactivity of NOS and the damage possibly caused by nitric oxide, nitrotyrosine immunostaining and the immunoreactivity of the different alveolar NOS isoforms were correlated. Nitrotyrosine had no association with

iNO therapy. In alveolar macrophages, nitrotyrosine immunostaining correlated weakly with the immunopositivity of all NOS isoforms (NOS1: $r_s = 0.4$, $p = 0.05$; NOS2: $r_s = 0.5$, $p = 0.01$; NOS3: $r_s = 0.5$, $p = 0.02$). The multiple regression test revealed an association between nitrotyrosine staining of AM and NOS activity of AM ($r = 0.58$, $p = 0.04$). Among the individual NOS scores, only NOS2 had a significant association with nitrotyrosine (regression coefficient = 0.42, $p = 0.01$). Nitrotyrosine present in the alveolar exudate or alveolar epithelium did not associate with any NOS scores in AM or in the alveolar epithelium.

5.2.6 Nitrotyrosine and NOS isoforms in healthy lung and in respiratory failure

In the alveolar lining and in the epithelium of the small peripheral airways of the five infants who did not die of respiratory failure, no immunoreactivity of nitrotyrosine was detectable. As compared to the infants with respiratory failure, with similar gestation at birth and with similar postnatal age (iNO cases and control cases 4, 7, 9 and 10), the frequencies of nitrotyrosine present in the alveolar exudates and the bronchiolar-alveolar epithelium were significantly lower in the healthy lung than in respiratory failure (0 of 5, vs. 5 of 8, $p = 0.02$). There were no other detectable differences in the distribution of nitrotyrosine or the NOS isoforms, as compared between the infants with healthy lungs and those with severe respiratory failure.

5.3 Exhaled NO measurements (III)

The mean gestational age at birth was 28 ± 1 wk in the group of premature infants and 38 ± 1 wk in the group of term/near-term infants. The mean birth weights were 863 ± 132 g and 3098 ± 417 g, respectively. The ventilation modes included synchronized intermittent mandatory ventilation, intermittent mandatory ventilation and continuous positive airway pressure. All preterm infants had RDS (n = 6) and were treated with exogenous surfactant (Curosurf® 100mg/kg). Three developed CLD (FiO_2 0.35, 0.90 and 0.24 at 36 wk of gestation). Two of the near-term infants had RDS. Three term infants had the meconium aspiration syndrome (one treated with surfactant) and one ductus-dependent tetralogy of Fallot. During the first week of life, none of the children had infection. However, three preterm infants and one near-term infant had late-onset sepsis (*Staphylococcus epidermidis* in two cases, *Staphylococcus homidis, E. coli*).

During the days of NO measurements, the preterm infants had higher MAP (7.9 ± 0.3 *vs*. 5.6 ± 0.7, $p = 0.014$) and end-expiratory pressures (4.5 ± 0.1 *vs.* 3.7 ± 0.3, $p = 0.016$) than the term/near-term infants. The preterm infants had similar spontaneous tidal volumes (4.0 ± 0.4 *vs.* 4.3 ± 0.6 ml/kg) but higher ventilator-driven tidal volumes (13.4 ± 2.3 *vs.* 5.8 ± 0.7 ml/kg, $p = 0.03$) than the term/near-term infants. The minute volumes were 288 ± 43 and 262 ± 20 ml/kg, respectively. The exhaled NO levels are shown summarised in Table 2.

Table 2. Summary of exhaled NO peak levels [median (range)], ppb.

Postnatal age, days	Preterm infants				Term/near-term infants			
	Expired maximal	Expired median	Nasal maximal	Nasal baseline	Expired maximal	Expired median	Nasal maximal	Nasal baseline
0 – 0.9	3.6 (1.5 – 6.3)	2.3 (0.5 – 5.0)	8.9 (3.5 – 94.5)	6.0 (1.4 – 30.0)	3.0 (2.1 – 12.5)	1.5 (0.7 – 11.0)	130.0 (7.1 – 490.0)	6.5 (2.0 – 50.0)
1.0 – 1.9	6.3 (2.6 – 44.0)	4.8 (0.5 – 36.0)	60.5 (13.5 – 539.0)	4.4 (2.5 – 27.0)	3.8 (2.0 – 6.3)	1.8 (0.2 – 2.5)	261.8 (75.0 - 914.0)	22.5 (10.0 – 55.0)
2.0 – 3.9	4.5 (1.2 – 15.5)	1.4 (0 – 14.2)	29.5 (4.0 – 110.0)	4.8 (2.0 – 34.7)	3.2 (2.5 – 13.5)	1.6 (0.8 – 12.5)	138.0 (76.0 – 425.0)	13.0 (3.0 – 80.0)
4.0 – 6.9	4.3 (2.0 – 26.8)	1.2 (0.2 – 22.0)	16.9 (3.5 – 990.0)	5.0 (2.0 – 20.8)	4.9 (3.5 – 22.8)	3.2 (0.9 – 20.8)	338.0 (6.4 – 630.0)	20.0 (3.2 – 130.0)

5.3.1 Expired NO levels

In terms of sufficient flow rate and expiratory time, the NO measurements were acceptable for practically all ventilator-induced breaths. In the preterm infants, 20 - 60% of spontaneous breaths were acceptable for analysis. Most of the unacceptable breaths had low flow rates (<1.7 ml/s).

In the preterm infants, the highest NO levels were measured during the second day of life. This trend was seen both in the maximal NO peaks recorded and in the median NO peak values. NO concentrations showed no significant correlation with the severity of respiratory failure (FiO_2, a/A-ratio, OI or VI), tidal volumes or minute ventilation. Of the three preterm infants who had the highest NO peaks at the age of 24 to 48 hours, two developed CLD. The infant with the highest exhaled NO concentration (maximum 44 ppb) manifested the most severe CLD. The infant with less severe CLD had the highest peak concentrations during the fifth postnatal day (maximum 26.8 ppb), while the third infant with the least severe CLD showed lower NO concentrations during the first week of life (maximum 5.8 ppb on the third postnatal day). Despite the higher tidal volumes of the preterm infants, the maximal NO peak concentrations of ventilator-induced breaths were higher than those of spontaneous breaths (3.0 ± 1.5 *vs.* 2.3 ± 1.4 ppb; p = 0.0007).

In the term/near-term infants, no significant correlations emerged between the maximal expiratory NO peak levels and the FiO_2, a/A-ratios, OI, VI or minute ventilation values. There was a significant positive correlation between the respirator-induced tidal volumes and maximal expiratory NO peak concentrations (r^2 = 0.4; p = 0.0001), but not in spontaneous breaths. The maximal NO peak concentrations of spontaneous breaths were higher than those of ventilator-induced breaths (1.7 ± 0.5 *vs.* 1.5 ± 0.6 ppb, p = 0.007).

5.3.2 Nasal NO levels

In the preterm infants, the highest NO peak levels were found during the second day of life. However, the baseline levels showed little change during the first week of life. In the term/near-term infants, the nasal NO peak levels were higher than in the preterm infants and tended to increase during the first week. The maximal nasal NO peaks were higher by one or two orders of magnitude than the maximal expired NO peak levels.

5.4 Very early nitric oxide treatment of premature infants (IV)

Four infants, who were not treated with iNO, died at the median postnatal age of 6 h (range 3.5 h – 11 days). The autopsy diagnoses were pneumonia and sepsis in two cases and pulmonary hypoplasia and hyaline membrane disease one case each.

Three infants treated with iNO had RDS and sepsis/pneumonia, while two had RDS and early-onset pneumonia (Figure 2). The pregnancy was complicated by PROM in each case. Serum C-reactive protein, airway IL-1β levels and NOS2 in the airway cells were low soon after birth. The respiratory failure was progressive, despite ventilation (HFOV in three cases) and surfactant therapy. Supra-systemic arterial pressures were confirmed by cardiac ultrasound examinations. Inhaled NO therapy was started at a mean postnatal age of four hours. In all cases, oxygen saturation increased, allowing a decrease in oxygen requirements within 10 minutes. The airway pressures could be decreased soon thereafter. Weaning from iNO was complete within 3 to 4 days. Methaemoglobin levels were low, except in case 3 with increased levels during day 0 (9.1% and 6.9%). The iNO dosage was decreased and the met-Hb level normalized. C-reactive protein, IL-1β and NOS2 increased within 24 to 48 h of birth. Platelet counts were above 90 x 10^9/l before and during the treatment.

Patients required ventilation for a mean of 12 days. The cases 1 and 2 had persistent *ductus arteriosus* and were treated with indomethacin. The cases 1, 2 and 5 required supplemental oxygen at the age of 28 days, the cases 1 and 5 at the post-conception age of 36 weeks (FiO_2 0.25 and 0.25), but none at 38 weeks. Case 2 had grade 1 intracerebral haemorrhage. Follow-up examinations at corrected ages from full-term (case 5) to 24 months (case 3) and 2 years (cases 1, 2 and 4) have so far revealed no respiratory problems, normal neurological findings and no evidence of sensory defects or cerebral palsy in any of the five children.

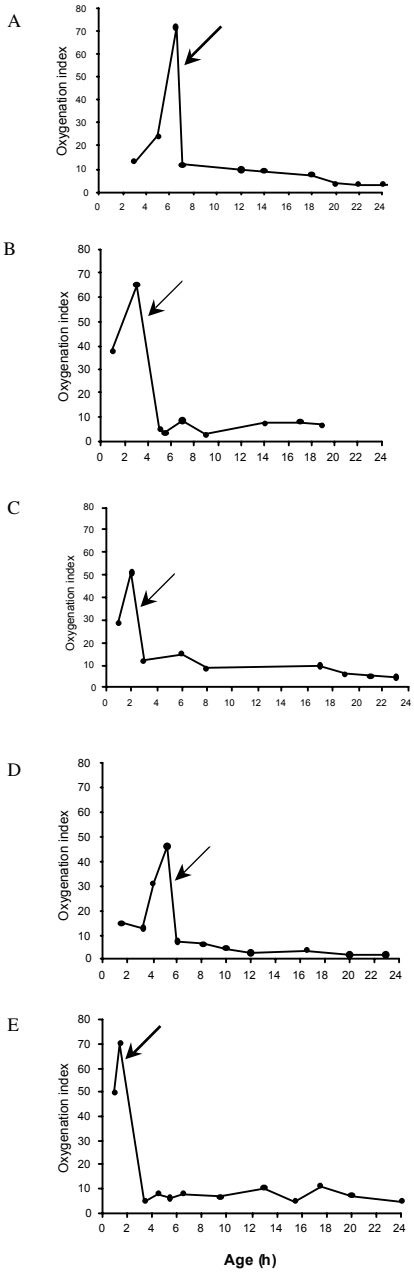


Fig. 2. The severity of respiratory failure before and after the onset of inhaled nitric oxide (arrows). The individual cases 1 to 5 are shown in panels A to E, respectively. The severity of respiratory failure is illustrated as the oxygenation index.

6 Discussion

We evaluated endogenous nitric oxide metabolism in the lungs of preterm and term infants suffering from severe respiratory failure, including a trial on the efficacy and safety of inhaled NO in premature infants very soon after birth.

6.1 Inflammatory NO production in early-onset pneumonia

NOS2 expression in alveolar macrophages in human newborns *in situ* is reported for the first time. Impairment of inducible NO production by pulmonary AM in fulminant early-onset neonatal pneumonia was identified. The low NOS2 immunoreactivity in AM in 92% of the cases with fatal early-onset pneumonia suggests a lack of an adequate response to a bacterial challenge. In the infants who died of non-infectious pulmonary disease or of pneumonia of later onset, AM were immunopositive for NOS2. The diminished NOS2 immunoreactivity was confirmed and extended by studying cells recovered from the airways in fulminant pneumonia and in appropriate controls.

The cause of the very low immunoreactivity of NOS2 in AM remains unknown. The data regarding the number and function of monocytes/macrophages of human neonates are limited. The number of macrophages is developmentally regulated in newborn primates (244) and humans (245). The number of AM was low at birth, increasing at the expense of interstitial macrophages during early neonatal transition in a rodent macrophage culture (246). Although the present lung specimens contained alveolar and interstitial macrophages without apparent differences between the infants with pneumonia and the controls of similar gestation and postnatal age (247), deficiency in the number of macrophages is not entirely ruled out.

The other possibility is that inflammatory cells did not adequately respond to the microbial challenge. The production of proinflammatory cytokines is required for the activation of NOS2 in murine (23, 104) and human (105) macrophages. Impaired activation of NOS2 by cytokines is supported by our finding of a lack of significantly increased IL-1β or TNF-α concentrations in the lung effluent from premature infants with fulminant early-onset pneumonia. The apparently non-infected infants exposed to IUI additionally had higher IL-1β than those with pneumonia. In adult animals, the induction

of NOS2 as a consequence of a microbial challenge takes place within 100 minutes (248). The current data suggest that AM from human neonates do not appropriately respond to a challenge of bacterial origin. This has been shown previously in human material for *Candida albicans* (249) and in animal models as well (250). Other impaired neonatal AM functions shown in animals include reduced opsonic receptor function (251), defects in the ingestion or killing of bacteria and impaired chemotaxis (252). Human newborn lung macrophages have a reduced capacity to restrict the intracellular growth of *Candida albicans* (253).

Our data suggest defects in the bactericidal activities of human neonatal macrophages. This evidence is consistent with the possibility that the induction of the NOS2 gene in AM is delayed or developmentally regulated. Indeed, it has recently been shown that the Toll-like receptors 4 and 2, the signalling receptors for gram-negative and gram-positive bacteria, respectively, are developmentally regulated. As studied in the murine lung, the expression levels of the Toll-like receptors 4 and 2 were very low in the premature foetus, increased towards term, and increased even further postnatally (254). It is possible that the deficient cytokine and NOS2 expression in premature infant with early-onset pneumonia is due to a lack of signalling receptors for bacterial toxins.

Immunohistochemical staining of human lung tissue with nitrotyrosine is an indicator of peroxynitrite, reflecting the reaction between NO and superoxide radicals (64). Apart from the availability of NO, the formation of nitrotyrosine is affected by activities that control the formation of superoxide. In the present study, NOS2 immunoreactivity in AM correlated with that of nitrotyrosine. Therefore, the low nitrotyrosine immunoreactivity in pulmonary AM from newborns with fulminant early-onset pneumonia was an additional indicator of low NO production. The fact that the alveolar exudate tended to contain less nitrotyrosine in the infants with fulminant pneumonia than in the controls, who mostly died of respiratory failure, further implies that pulmonary NO toxicity was not likely to contribute to the progressive course of fulminant early-onset pneumonia.

Lung surfactant has been shown to enhance NO production in AM (255). In particular, the surfactant-associated lectins, SP-A and SP-D, bind to specific microbes and augment phagocytosis by AM (128). Congenital absence of SP-A in mice predisposed to generalized GBS infection (256). In the present study population, which mainly consisted of premature infants, many of whom had GBS sepsis, the additional finding of low SP-A suggests a severe deficiency in innate immunity. Our finding, that the non-septic infants with intrauterine infection had significantly higher IL-1β and SP-A than those with pneumonia, is consistent with the possibility that IL-1 derived from amniotic fluid up-regulates surfactant components (257) and may improve other host defense functions as well.

A significant, although unknown, fraction of NO produced in lungs is likely to be eliminated into exhaled air (138). Thus, the serum and urine nitrite/nitrate levels previously measured from elder infants (124, 125) reflect mainly extrapulmonary NO production. The present results on pulmonary NO production are not in disagreement with the concept of excessive NO production in septic shock.

Specific lung cells also contain constitutive NOS activity. NOS1 and NOS3 contribute to the NO output. They may also be affected by infections or by substrate availability for NO synthesis. According to experimental studies, endotoxin down-regulates constitutive

NOS (258), whereas in non-infectious forms of pulmonary hypertension endothelial NOS3 may be increased (259).

6.2 Pulmonary NOS expression in respiratory failure

The expression of NOS isoforms in the lungs of the infants was demonstrated. Inhaled NO, given in severe respiratory failure, was associated with an increase of the immunostaining of airway epithelial NOS2, and it had no effects on the immunostaining of NOS1 and NOS3 in any of the pulmonary cell types studied.

Since the present specimens were from autopsies, it is possible that some of the findings are due to post-mortem changes. However, as indicated in the following discussion, the cellular distribution of nitrotyrosine and NOS immunoreactivity is consistent with the observations on experimental animals that are premature or have serious lung damage. In the subjects without lung disease, nitrotyrosine accurately reflected severe alveolar and bronchial disease. In series I, the immunoreactivity of nitrotyrosine and NOS2 revealed similar changes both in the lung specimens obtained from autopsies and in the airway specimens recovered from ventilated infants. Another limitation could be the heterogeneity of the study population. The paired controls were matched as well as possible.

The respiratory epithelium contained all the three NOS isoforms. NOS expression increased towards the proximal airways. The immunoreactivity of NOS2 at the bronchiolar epithelium was stronger in the iNO-treated group than in the controls. Similar trends in the staining of NOS2 at the bronchial epithelium and bronchial smooth muscle cells were evident, too. These observations suggest that exogenous NO enhances the immunoreactivity of NOS2. This finding indicates that NO had a local intracellular effect. The significance of this effect remains to be studied.

Regardless of respiratory failure, all the NOS isoforms were detected along the airway epithelia. The roles of NOS in the epithelium of the respiratory tree remain unknown. Different isoforms may participate in NO-requiring physiologic functions, and additionally have pathogenetic or compensatory roles in severe respiratory failure.

The vascular endothelia were positive for the NOS isoforms without significant developmental trends and without any influence of the respiratory failure. According to the prevailing theory, pulmonary vascular endothelium has high basal expression of NOS3, catalysing the formation of NO required for the activation of cGMP in vascular smooth muscle (43).

Both airway and vascular smooth muscle revealed faint immunostaining of the three NOS isoforms. Again, neither postnatal age nor the severity of the respiratory failure correlated with the expression of NOS. Exogenous NO tended to increase NOS2 expression in bronchial smooth muscle cells, possibly by the same mechanisms as in the epithelium.

Alveolar macrophages showed immunolabeling of all NOS isoforms. In series I, the NOS2 immunoactivity of alveolar macrophages was lower in the infants with fulminant pneumonia shortly after birth than in the controls with severe, non-infectious respiratory failure. However, in the postnatal lung, activation of AM in pneumonia was evident. In

series II, the often moderate to low degree of immunostaining of NOS2 in AM could be due to the lack of infection or to the non-responsiveness of AM to microbial products. However, NOS2 was associated with the formation of nitrotyrosine. In the present study of infants with respiratory failure, there was detectable NOS1 and NOS3 immunostaining in AM, suggesting the presence of constitutive NO production.

6.3 Pulmonary nitrotyrosine in respiratory failure

Inhaled NO (1 - 60 ppm) during 0.1 - 16 days was not associated with increased nitrotyrosine immunopositivity compared to the controls, suggesting that iNO treatment did not increase the toxic oxidation products of NO. The epithelium of the distal airways and the extracellular spaces of the alveoli in healthy lungs did not contain nitrotyrosine immunostaining, whereas such staining was frequently evident in progressive respiratory failure.

Immunostained nitrotyrosine represents a protein that has nitrosylated tyrosine residues, which may disturb the function of a specific protein (44). The detection of nitrotyrosine provides evidence of the generation of reactive nitrogen species (260) and is considered a biomarker of the toxic NO-ONOO-pathway (261). Previously, nitrotyrosine has been detected in ARDS (67) and in other severe lung diseases (68, 69, 262). Inhaled NO at 20 ppm or less apparently increased the levels of 3-nitrotyrosine in bronchoalveolar lavage fluid from patients with ARDS (263). However, in term newborns, iNO therapy (≤20 ppm for 1 to 4 days) was not associated with an increase in nitrotyrosine-tagged proteins in airway specimens (238). The current results extend these findings.

In the present study, nitrotyrosine was detectable in alveolar tissue. These patients had been suffering from severe respiratory symptoms and hypoxemia and had therefore required high concentrations of supplemental oxygen. Some were exposed to pulmonary ischemia and re-perfusion. Under these conditions, the formation of superoxide is likely. However, in newborn piglets, 50 ppm iNO together with hyperoxia did not influence on the degree of lung injury (264). On the other hand, iNO at 100 ppm has been shown to increase the survival of rats with hyperoxia (265). Inhaled NO at 20 ppm with concurrent hyperoxia did not increase intra- or intercellular nitrotyrosine in a mouse model, either (266) According to the present and previous evidence (72), NO and superoxide toxicity is a possible mechanism causing severe lung disease in infants. However, administration of iNO in severe respiratory failure was not associated with any evidence of increased NO toxicity.

NO serves as an antioxidant, protecting against oxidant stress. Although the formation of peroxynitrite may be enhanced in lungs exposed high doses of inhaled NO, nitrotyrosine was not increased in the present group infants. Most iNO-treated and control infants who died of severe respiratory failure had detectable nitrotyrosine in the proteinous alveolar exudate. On the other hand, iNO increased NOS2 expression mainly in the proximal airway without causing an increase in nitrotyrosine. No significant relationship between the dosage of iNO and the nitrotyrosine or NOS2 immunoreactivities could be established. The significance of this finding remains unclear.

Since NOS2 is mostly regulated by inflammatory agonists and NO may reportedly cause an inflammatory reaction (108), the documented increase in NOS2 as an index of airway toxicity cannot be excluded.

6.4 Expired NO in mechanically ventilated newborns

The present study demonstrated a novel method for measuring nitric oxide concentrations in the upper and lower airways of mechanically ventilated infants. Nasal NO concentrations were distinctly higher than those in expired air. Neither the length of gestation nor neonatal age explained the wide variation of the NO levels.

Several factors complicate the measurements of exhaled NO from the lower airways. Inhalation of nasal NO is one source of variation. In the present setting, nasotracheal intubation prevented the entry of nasal NO into the lower airways. Traces of NO gas are ubiquitously present in ambient air. Hospital compressed air also contained low levels of NO, which were measured before and after the patient recordings. There was no correlation between the lower airway and hospital air NO levels. To measure strictly pulmonary NO excretion, inhalation of NO-free air or a NO-absorbing filter between the intubation tube and the respiratory circuit would be required. NO-free air intervention was not possible due to the severe respiratory compromise of these vulnerable patients. The available charcoal filter did not quantitatively remove NO (267). In the results presented, the hospital air concentrations of NO have been subtracted from the exhaled NO concentrations, resulting in concentrations lower by a mean of 29.4 ± 2.6% than those actually recorded (268).

We propose that the NO peak levels recorded in exhaled air represent the concentrations of NO in the lower airways. Inhaled NO may be re-expired, taken up by the lung or degraded; the half-life of NO in humidified air at 37°C is about 10 s. In addition, endogenous NO in expired air may be diluted by gases from the ventilatory circuit. As tested in the lung model, the patient-generated NO peaks during short expiration (•0.2 s) and low-flow conditions (•1.7 ml/s) were excluded from the analysis, because the NO levels were underestimated due to dilution. As a result of the low flow rates, only 20 to 60% of spontaneous breaths could be accurately measured in small preterm infants.

Exhaled NO levels depend on the expiratory flow rate. As measured during spontaneous ventilation in adults, NO values increased 35-fold as flow decreased 400-fold (269). Mechanical ventilation may also affect the NO output. We determined the respiratory flows and pressures as well as the origin of a single breath. In small preterm infants, respirator-induced breaths had significantly higher volumes and flow rates than patient-originated breaths. Their peak NO concentrations in respirator-induced breaths were higher than those in spontaneous breaths, although tidal volumes were higher and the fractions of autoinhaled NO lower. In the term/near-term infants, the respirator-induced tidal volumes correlated positively with expired NO levels. Shear stress has been described to enhance vascular NO production in endothelium (167). Likewise, airway epithelium could produce more NO as a reaction to mechanical stretching and shear forces across the airways in lung disease. We propose that shear stress due to

inappropriately high tidal volumes may increase NO production. This possibility remains to be studied further.

The NO levels from the lower airways of the small preterm infants tended to increase after birth and peaked at 24 to 48 h of life. The term/near-term infants showed no consistent trends in their NO levels during the first week. In the present study, the exhaled NO concentrations varied considerably between the individual patients. The causes of major variation and the possible relationship of NO excretion with the pathogenesis of neonatal lung disease remain to be studied.

6.5 Nasal NO in mechanically ventilated newborns

In the upper airways of the preterm infants, the maximal NO peak concentrations showed a similar postnatal pattern as in the lower airways. The highest values were measured at 24 to 48 h of life. On the other hand, the maximal nasal NO peak levels of the term/near-term infants showed a trend towards a postnatal increase during the first week of life. Previously, the highest levels of endogenous NO have been measured from the paranasal sinuses with an age-dependent increase in keeping with sinus pneumatization (155). Schedin *et al*. measured post-occlusive NO levels from the upper airways of healthy, spontaneously breathing term and preterm infants (146, 147). Term newborns produced high nasal NO concentrations, which increased by 30% during the first 24 h. The nasal NO level was significantly associated with gestational age at birth as well. The present measurements, using the non-intubated nostril of ventilated infants with lung disease, were consistent with the findings on healthy infants (146). Since expiratory flow affects on the NO levels, the results might have been different if the infants had been orotracheally intubated. However, for spontaneous breaths, occlusion of the nostril is necessary for standardization of the measurements. Nasotracheal intubation is also an occlusive situation because there is no respiratory flow through the free nostril.

The physiological role of upper airway NO is unknown. Autoinhalation of nasal NO has been proposed to modulate pulmonary hemodynamics (159), though exhaled NO did not correlate with vascular endothelial function (270). Microbial exposure induces NO synthesis (156), serving as a component of the innate immune response, as suggested in series I. The high NO levels in the airways of the small preterm infants during the second day of life may reflect adaptation to postnatal life or the severity of the pulmonary disease.

6.6 Very early iNO to premature infants with intractable respiratory failure

The very low birth weight infants with intractable respiratory failure and persistent pulmonary hypertension responded strikingly to the administration of nitric oxide, whereas all the four infants treated with the conventional therapy died.

In the randomised trials demonstrating a small response, the inhaled NO therapy was started at a later age for a less severe respiratory failure than in the present cases (mean OI 20 *vs*. 60; a/A-ratio <0.10 *vs.* median a/A-ratio 0.04) (222, 223). The time after birth, the dosage of iNO and the degree and aetiology of respiratory failure may influence the efficacy. According to series I, pulmonary NO may be deficient after a very premature birth and in lung hypoplasia (218). In the present cases, infection and pulmonary hypoplasia (four had PROM for at least two weeks) may have contributed to severe respiratory failure. We propose that inhaled NO supports compromised pulmonary adaptation in cases of early-onset pneumonia.

Infection has been regarded as a contraindication for iNO (271). In the present study, NO was given despite suspected infection, since in series I, early-onset pneumonia was associated with low activity of inducible NO synthase and with no evidence of NO toxicity. Low cytokine levels and very low NOS2 immunoreactivity were confirmed in the airway specimens from these patients. However, excessive NO production is evident during advanced post-neonatal septic shock (272). In the series I and IV, NOS2 and IL-1β increased in the cells from airway specimens during recovery. The present observations do not assess the safety of iNO at the late stages of neonatal infection. However, in series IV, with the exception of transient methaemoglobinemia in one case, no other side effects were evident in early-onset pneumonia.

7 Conclusions

The production of nitric oxide and peroxynitrite was diminished in newborn infants with fulminant early-onset pneumonia. The premature infants also had low pulmonary proinflammatory cytokines, low pulmonary C-type lectin and diminished NOS2 expression by alveolar macrophages, which may have predisposed them to severe pneumonia. During the period of recovery, these parameters were up-regulated. However, deficient production due to immaturity of the pulmonary immune system rather than excess pulmonary NO was associated with severe symptoms in fulminant early-onset pneumonia.

Inhaled NO, up to 60 ppm for 0.1 to 16 days, did not increase nitrotyrosine in the alveolar tissue, suggesting a lack of toxicity of iNO due to oxidant injury. However, inhaled NO, during severe respiratory failure, increased NOS2 immunoactivity in bronchiolar epithelium and adjacent tissue, suggesting that exogenous NO increases NOS2 protein by an unknown mechanism. While caution about potential pulmonary toxicity remains, the present result further justifies therapeutic trials on iNO in respiratory failure.

A novel method allowing studies of airway NO levels and their association with neonatal pulmonary disorders in mechanically ventilated newborns was established. A difference in the patterns of NO excretion between preterm and term/near-tem infants was observed during the first week of life. Exhaled NO in the infants with respiratory failure probably reflected the summation of baseline NO production, induced production as a consequence of inflammatory activity, mechanical stretch or other activities. Endogenous NO may be consumed in pro- and antioxidative reactions, and the rate of these reactions may affect the levels of exhaled NO.

The finding of diminished NO production in the pulmonary AM in series I and the lack of obvious pulmonary toxicity of iNO in series II allowed a pilot intervention study on inhaled NO for premature infants. Very early iNO therapy acutely improved oxygenation in a select group of premature infants with intractable respiratory failure. However, more research and randomised controlled trials are required to confirm the efficacy and safety of iNO in premature infants very soon after birth.

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