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NITRIC OXIDE IN EXHALED GAS

Studies on physiological regulation and measurements in infants and children

by
Andreas Artlich

Stockholm 1999

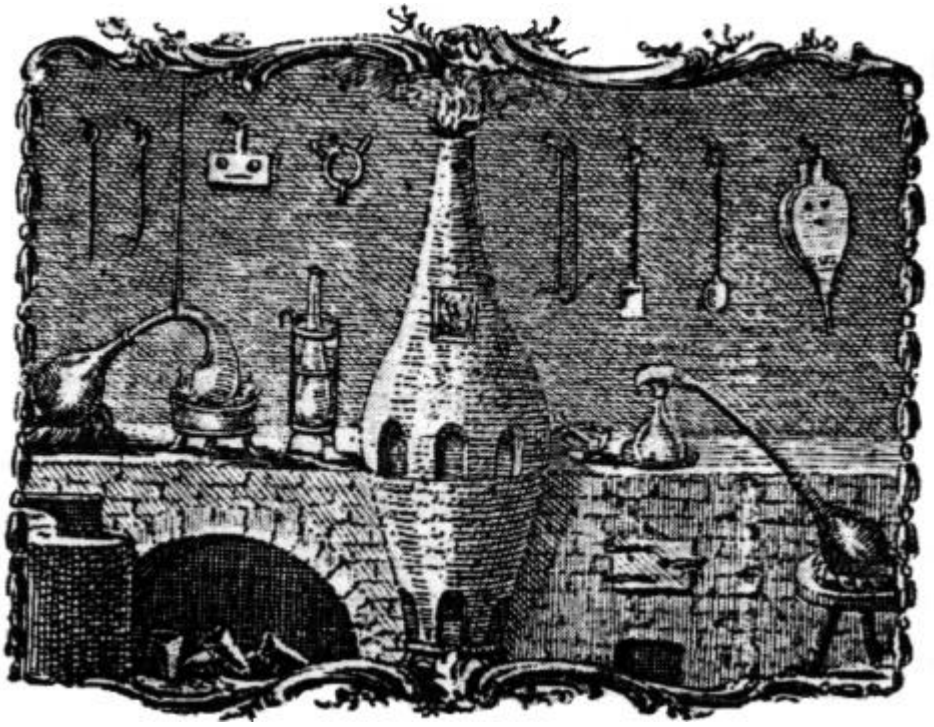
To Monika

for her loving support and for gently reminding me of the

secrets of life beyond science and clinical paediatrics.

«… and this is the reward I have received for my work, and which gives me real pleasure, so I can by no means keep to myself.
This also is the purpose and no other why I make this work known to my readers.»

Carl Wilhelm Scheele (1777) [Chemical treatise on air and fire.]



« ... und dieses ist der Lohn so ich fuer meine Arbeit erhalten, und welche mir ein rechtes Vergnuegen verursachet, so ich unmoeglich vor mir allein behalten kan. Dieses ist auch die Absicht und keine andere warum ich meinen Lesern diese Arbeit bekandt mache. »

Carl Wilhelm Scheele (1777) Chemische Abhandlung von der Luft und dem Feuer.
Uppsala, Magnus Swederus and Leipzig, Siegfried Lebrecht Crusius.

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ABSTRACT

Nitric oxide (NO) is found in the exhaled gas of humans, starting from birth, and is increased in adult patients with bronchial asthma. This study assessed whether the measurement of NO in exhaled gas is technically feasible in infants and children. We studied a possible difference of exhaled NO levels between asthmatic children and healthy controls. Optimising existing sampling techniques, a link between clinical severity and levels of exhaled NO was sought. Adapting the sampling technique to the specific needs of infants, tidal analysis of inhaled and exhaled NO concentrations was performed. Finally, regulatory aspects of pulmonary NO synthesis were studied in an established rabbit model.

We found that NO in exhaled gas can be reliably measured in newborn infants and children and distinguished from nasally derived NO. In spite of comparable lung function parameters orally exhaled NO was significantly increased in asthmatic children in comparison with healthy controls. Even higher increases were seen in asthmatic children with recent clinical symptoms of airway obstruction, suggesting that exhaled NO measurements may be an early signal of asthmatic airway inflammation and useful to monitor the effectiveness of anti-inflammatory treatment. In newborn infants the range of autoinhaled NO concentrations made biological effects of NO in the lower respiratory tract conceivable. In the rabbit experiments adrenoceptor stimulation and high frequency oscillatory ventilation significantly increased pulmonary NO production. These findings are likely relevant to the understanding of the circulatory adaptation to extrauterine life. Increases of pulmonary NO production induced by mechanical stretch might influence bronchial function and ventilation perfusion matching.

Key words: adrenaline, adrenoceptor, artificial ventilation, asthma, carbon dioxide, catecholamines, chemiluminescence, child, cilia, exhaled gas, focal adhesion complex, high frequency oscillatory ventilation, lung, mechanotransduction, newborn, nitric oxide, glucocorticosteroids, stretch

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ABBREVIATIONS

AMP	adenosine-5´-monophosphate
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
BHR	bronchial hyper-responsiveness
CLD	chemiluminescence detector
COPD	chronic obstructive pulmonary disease
ECP	eosinophilic cationic protein
eNOS	endothelial NOS
FAC	focal adhesion complex
GC-MS	gas chromatography – mass spectroscopy
HMOX1	haeme oxygenase 1
IFN-γ	interferon γ
IL	interleukin
IMV	intermittent mandatory ventilation
iNANC	inhibitory non-adrenergic non-cholinergic neurotransmission
iNOS	inducible NOS
L-NAME	N^{ω}-nitro-L-arginine methylester
L-NMMA	N^{G}-monomethyl-L-arginine
NFκB	nuclear factor κB
NOS	nitric oxide synthase
nNOS	neuronal NOS
NO	nitric oxide
PDE	phosphodiesterase
PEF	peak expiratory flow rate
PLC	phospholipase C
PVR	pulmonary vascular resistance
RNS	reactive nitrogen species
ROS	reactive oxygen species
RTLF	respiratory tract lining fluid
TNF-α	tumour necrosis factor α
URTI	upper respiratory tract infection

VEGF	vascular endothelial growth factor

1. INTRODUCTION – BACKGROUND

The evolution of nitric oxide (NO) as a signalling and effector molecule in biological systems dates back millions of years. Synthesis of NO from the aminoacid L-arginine can be detected in a variety of tissues even in arthropods and molluscs and is accomplished by a number of isoenzymes of NO synthase (NOS) (Johansson & Carlberg, 1995). Three isoforms of NOS can be differentiated in mammalian species: Neuronal NOS (nNOS or type I NOS), inducible NOS (iNOS or type II NOS) and endothelium specific NOS (eNOS or type III NOS) which in humans are genetically coded on chromosomes 12, 17 and 7, respectively (Knowles & Moncada, 1994). All NOS isoenzymes are complex calmodulin-dependent homodimers resembling NADPH-cytochrome-P450 reductase (Schmidt *et al.*, 1993). Endothelial NOS is a haemoprotein which requires several cofactors (tetrahydrobiopterin, FAD / FMN, NADPH) for the oxidation of the L-arginine guanidino group to produce NO. Haemodynamic (shear stress and flow), hormonal (e.g. oestrogen), paracrine (e.g. growth factors) and pharmacological (e.g. acetylcholine) stimuli increase endothelial cell calcium concentration, thereby enhancing calmodulin binding to eNOS and increasing NO production. In contrast to the eNOS and nNOS isoenzymes, which are constitutively expressed and Ca^{2+}-dependent, the expression of iNOS is induced by lipopolysaccharide and proinflammatory cytokines (Lincoln *et al.*, 1997). Activation of iNOS is not dependent on free intracellular Ca^{2+} ions and produces amounts of NO that exceed the output of eNOS by a factor of more than 1000 (Änggård, 1994). Several variants of nitric oxide synthases are known and are formed by variable transcription and alternative splicing of mRNA (Wang *et al.*, 1999). Repeats of iNOS-like gene sequences gene are found on chromosomes 14 and 17, but the significance of this finding is unknown (Xu *et al.*, 1995; Park *et al.*, 1997). Enzymatic NO synthesis is complemented by non-enzymatic NO formation, a concept which has recently gained interest even in humans (Weitzberg & Lundberg, 1998). At a low pH, i.e. in an acidic/reducing environment, the nitrite ion (NO^{2-}) will be converted to nitrous acid (HNO_2) and then various nitrogen oxides including NO (Kelm & Yoshida, 1996; Weitzberg & Lundberg, 1998).

Once formed, NO diffuses away from the producing cell and activates soluble guanylate cyclase in the cytoplasm of its target cell by binding to its haeme group leading to increased formation of cGMP. Having exerted its effect mostly via activation of cGMP-dependent protein kinase (Schmidt *et al.*, 1993), cGMP is hydrolysed by cGMP-specific phosphodiesterase (PDE1 and PDE5) thus limiting the biological effect of NO. As an alternative to cGMP-mediated signal transduction, there is evidence that NO can exert direct effects on ion channels in vascular smooth muscle (Bolotina *et al.*, 1994; Shuttleworth & Sanders, 1996). Finally, high local concentrations of NO can, by inactivation or interaction with cellular proteins (Schmidt *et al.*, 1993), inhibit cell growth (Nussler & Billiar, 1993) and take part in non-specific immune defence against bacteria (Nathan & Hibbs, 1991), protozoa (Wei *et al.*, 1995) and viral agents (Croen, 1993; Sanders, 1999).

The interactions of NO with other biomolecules and the resulting effects in the cardiovascular system, the gastrointestinal, urogenital and immune system, in neuronal signalling and in the pathogenesis of common diseases (e.g. asthma, diabetes, arterial hypertension) are legion and have extensively been reviewed (Änggård, 1994; Lincoln *et al*., 1997). The physiological role of nitric oxide (NO) in the healthy lung and in various pulmonary diseases is increasingly being appreciated and will be discussed in more detail.

1.1. PHYSIOLOGICAL ROLE OF NITRIC OXIDE IN THE LUNG

Pulmonary circulation

Soon after the discovery of an endothelial-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980), subsequently identified as NO (Ignarro *et al*., 1987; Palmer *et al*., 1987; Furchgott, 1988), and the disclosure of its major function as a regulator of peripheral vascular tone (Aisaka *et al*., 1989; Rees *et al*., 1989), the relevance of these findings was studied in the pulmonary vascular bed. Ample experimental evidence suggests that NO can affect baseline pulmonary vascular tone and contributes to the pathogenesis of both acute and chronic hypoxic pulmonary hypertension (for review cf. Hampl & Archer, 1997). In healthy humans the importance of nitric oxide in the maintenance of pulmonary vascular tone has been illustrated by the effect of NOS inhibition using N^G-monomethyl-L-arginine (L-NMMA) (Stamler *et al*., 1994; Albert *et al*., 1997; Kiely *et al*., 1998). Both systemic and local application of this NOS inhibitor into the segmental pulmonary artery led to significant increases in pulmonary vascular resistance (PVR), suggesting that normal basal PVR is maintained by a continuous local NO production specific to the lung (Stamler *et al*., 1994; Albert *et al*., 1997; Cooper *et al*., 1996).

The physiological role of NO in the regulation of pulmonary vascular tone is accentuated during the vascular adaptation to extrauterine life (Shaul, 1995; Fineman *et al*., 1997). This has elegantly been demonstrated in lambs by the administration of a NOS inhibitor immediately before delivery by Caesarean section (Abman *et al*., 1990). In comparison with control animals the postnatal rise in pulmonary blood flow 1h after birth was significantly reduced in the treated animals (Abman *et al*., 1990). Chronic nitric oxide inhibition in utero produces persistent pulmonary hypertension in newborn lambs (Fineman *et al*., 1994).

The understanding of the crucial role of NO in the regulation of pulmonary vascular tone has culminated in a new treatment option - the inhalation of gaseous NO - to alleviate pulmonary hypertension in experimental and clinical settings. In the few years since the introduction of this new concept by Frostell and Zapol, first in lambs (Frostell *et al*., 1991) and later in healthy human

volunteers (Frostell *et al*., 1993), NO inhalation therapy has revolutionised the care of sick neonates with persistent pulmonary hypertension (PPHN) (Kinsella *et al*., 1992; Roberts *et al*., 1997) and has extensively been studied in adult patients with acute respiratory distress syndrome (ARDS) (for review cf. Troncy *et al*., 1997).

Bronchial tone

The relaxing effect of NO, organic nitrates and other NO-donating agents on vascular smooth muscle (Hobbs & Ignarro, 1997) has kindled interest in possible similar effects on airway smooth muscle (Katsuki *et al*., 1977). Indeed, endogenous NO affects basal bronchial tone (Nijkamp *et al*., 1993) and partly antagonises bronchoconstriction in response to vagus nerve stimulation, bradykinin, histamine or cold air in guinea pigs, rats, rabbits and humans (Belvisi *et al*., 1991; Belvisi *et al*., 1992; Lei *et al*., 1993; Nijkamp *et al*., 1993; Figini *et al*., 1996; Dewachter *et al*., 1997; Kanazawa *et al*., 1997a; Yoshihara *et al*., 1998; Homma & Irvin, 1999). In guinea pigs NO functions as a neurotransmitter mediating bronchodilator responses in the inhibitory branch of the non-adrenergic non-cholinergic peripheral nervous system (iNANC) (Li & Rand, 1991) and regulates the magnitude of cholinergic bronchoconstriction (Belvisi *et al*., 1991). These findings are particularly relevant to the human because, in contrast to other species, human airways lack a functional sympathetic innervation. Thus the prominent neural bronchodilator pathway is the iNANC pathway in which NO is the endogenous neurotransmitter (Bult *et al*., 1990; Belvisi *et al*., 1992). The iNANC system may be the functional basis for the observation that endogenous NO alleviates antigen-induced bronchoconstriction in sensitised animals (Persson *et al*., 1993a). Studies on patients with mild allergic asthma have however not revealed a similar role of endogenous NO in the human (Taylor *et al*., 1998a).

Analogous to trials of inhaled NO gas to treat pulmonary hypertension, possible bronchodilating effects of NO inhalation have been evaluated. 5-30 ppm NO gas or an aerosolised NO-donating compound relieve induced bronchoconstriction in a dose dependent manner in guinea pigs, rabbits, dogs and pigs (for review cf. Dupuy & Frostell, 1997). In the human inhalation of 80 ppm NO is mildly potent to alleviate methacholine-induced bronchoconstriction but does not change baseline airway conductance (Högman *et al*., 1993; Sanna *et al*., 1994). Consistently, therapeutic trials of NO inhalation to relieve bronchospasm in asthmatic patients have been largely disappointing in both adults (Högman *et al*., 1993; Högman *et al*., 1994; Kacmarek *et al*., 1996) and children (Pfeffer *et al*., 1996). These clinical studies support the general notion that equivalent doses of NO are less active bronchodilators than vasodilators (Katsuki *et al*., 1977; Gruetter *et al*., 1989).

Bronchial circulation

Extensions of the discovery of the vasodilator properties of NO in the pulmonary circulation have been studies on the effects of NO on the other vascular bed of the lung, namely the bronchial circulation. Inhalation of exogenous NO gas at high concentrations (20-300 ppm) increases bronchial blood flow and decreases bronchial vascular resistance in a dose-dependent manner (Alving *et al*., 1993a; Charan *et al*., 1997).

Fluid homeostasis

Various studies indicate that endogenous NO can thwart the balance between absorptive and secretory mechanisms towards a net absorption of fluid from the lower airways. NO reduces lung liquid production in fetal lambs (Cummings, 1997; Kabbani & Cassin, 1998) and influences pulmonary transmembrane chloride currents in bronchial epithelial cells (Kamosinska *et al*., 1997). It also acts as an endogenous inhibitor of both basal and neurogenic mucus secretion (Ramnarine *et al*., 1996) and suppresses plasma exsudation from the subepithelial microcirculation (Erjefält *et al*., 1994). In lung parenchyma NOS inhibition and blockage of β-adrenoceptors equally enhance neurogenic plasma leakage (Liu *et al*., 1994), again suggesting a suppressive effect of endogenous NO on plasma exsudation. In contrast to that, NO mediates microvascular leakage induced by intravenous application of inflammatory mediators (Kageyama *et al*., 1997) and is capable of enhancing neurogenic plasma leakage in the airways (Kuo *et al*., 1992; Ohuchi *et al*., 1998). Addressing this discrepancy, a recent study has suggested that NO suppresses plasma leakage under basal conditions but that induction of iNOS enhances leakage via increased formation of NO (Bernareggi *et al*., 1997).

Interactions with surfactant

In addition to known molecular interactions of NO and surfactant, particularly under hyperoxic conditions (Robbins *et al*., 1995; Hallman, 1997), there is evidence of regulatory feedback between NO and surfactant formation. Pulmonary surfactant enhances NO formation when given to surfactant-deficient piglets (Yu *et al*., 1997) and has been shown to increase basal NO production in unstimulated rat alveolar macrophages (Miles *et al*., 1998). Bioactive surfactant components however inhibit LPS-induced NO production in alveolar macrophages (Miles *et al*., 1999). Conversely, NO has been shown to inhibit surfactant synthesis by human type II pneumocytes (Vara *et al*., 1995).

Ciliary function

When ciliary beating is stimulated with salbutamol, a β–adrenoceptor agonist, NO release from cultured airway epithelial cells increases immediately (Tamaoki *et al*., 1995a). The stimulation of ciliary motility by cilia agonists such as the β–agonists isoproterenol and salbutamol, bradykinin, substance P

or proinflammatory cytokines is markedly affected by NOS inhibition (Jain *et al*., 1993; Jain *et al*., 1995; Tamaoki *et al*., 1995a). These findings suggest that NO is involved in the stimulation of ciliary motility. In concordance with that, patients with ciliary dysfunction exhibit a marked reduction of endogenous NO in both upper and lower airways (Lundberg *et al*., 1994c; Karadag *et al*., 1999). The aerosolic administration of the NO-precursor L-arginine to these patients results in a significant increase of NO concentration in nasal gas, nasal ciliary beating frequency and nasal mucociliary clearance (Loukides *et al*., 1998b). It is at present unclear how the genetically defined defects in the motor mechanism of cilia (McKusick, 1986) can explain the severe reduction of NO in exhaled gas.

Growth and development

A recent study in a strain of rats prone to develop pulmonary hypertension suggests that an intact pulmonary production of endothelial NO may be important for a proper alveolarization of pulmonary tissue during lung development (Kim *et al*., 1998). Endogenous NO also modulates the pulmonary gene expression of vascular endothelial growth factor (VEGF) and of VEGF receptors in response to hypoxia and is thus important in reactive blood vessel remodelling (Tuder *et al*., 1995).

Redox interactions

Free radicals, e.g. the superoxide anion ($O^{\bullet}_{2}^{-}$), the hydroxyl radical $^{\bullet}OH$, $NO^{\bullet}$ and nitrogen dioxide $NO^{\bullet}_{2}$, contain unpaired electrons making them competent to react with other radicals and, if sufficiently reactive, with non-radical molecules (Chabot *et al*., 1998). Free radicals are short-lived and form more stable metabolites commonly referred to as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Chabot *et al*., 1998; Patel *et al*., 1999). In biological systems the major source of RNS is NO (Patel *et al*., 1999). One of the major routes of RNS formation is the reaction of NO with the superoxide anion ($O^{\bullet}_{2}^{-}$) to form peroxynitrite ($ONOO^{-}$) (Beckman & Koppenol, 1996). Peroxynitrite is reactive with all major classes of biomolecules (O'Donnell *et al*., 1999; Patel *et al*., 1999). It can induce bronchial hyper-responsiveness (Sadeghi-Hashjin *et al*., 1996) and mediates some of the aspects of acute lung injury (Royall *et al*., 1995). On the other hand interaction with thiols can lead to formation of S-nitrosothiols, e.g. S-nitrosoglutathione, which have NO-donating properties and biological activity as bronchodilators (Gaston *et al*., 1993; Patel *et al*., 1999). Hence NO and its metabolites can both act as mediators of signal transmission and as chemical toxicants. During normal metabolism the production of free radicals, which are constantly being formed during the oxygenation of haemoglobin and through leakage from electron transport chains, is kept to a minimum by an efficient system of antioxidant defence (Winterbourn, 1996). An oxidant-antioxidant imbalance involving alterations of NO metabolism is important in the pathogenesis of pulmonary diseases such as asthma (Rahman *et al*., 1996; De Raeve *et al*., 1997), cystic fibrosis (Winklhofer-Roob, 1994) and ARDS

(Chabot *et al*., 1998). In patients with mild asthma a reduction of anti-oxidants (Vitamin C & E) in both BAL and bronchial washes has been reported (Kelly *et al*., 1999). More evidence suggesting altered redox metabolism in severe asthma lies in the recent finding that S-nitrosothiols, endogenous metabolites of NO with bronchodilator activity, are decreased in tracheal secretions of asthmatic children in respiratory failure (Gaston *et al*., 1998).

Pulmonary inflammation

All cellular components of pulmonary inflammation can produce NO, e.g. pulmonary macrophages, airway epithelial cells, mast cells, lymphocytes, granulocytes (neutrophils, eosinophils, basophils) and fibroblasts (Pendino *et al*., 1993; Asano *et al*., 1994; Gaston *et al*., 1994; Punjabi *et al*., 1994; Robbins *et al*., 1994b; Willis *et al*., 1994; Warner *et al*., 1995; Robbins *et al*., 1997; Miles *et al*., 1998). Increased NO production in one or more of these cell types has a number of functional implications. From a general point of view, high local concentrations of NO can mediate cytotoxicity against bacteria (Moncada *et al*., 1991; Dupuy & Frostell, 1997), parasites (Dupuy & Frostell, 1997) and viral agents (Nathan & Xie, 1994; Powell & Baylis, 1995; Sanders, 1999). Since the position of the lung in the circulation makes it important as an immunological filter (Moncada *et al*., 1991), the role of in non-specific defence probably applies to the lung as well (Blomqvist *et al*., 1993; Dupuy & Frostell, 1997). More specific interactions between NO and other bioactive molecules are required to inhibit leukocyte adhesion and chemotaxis or mast cell-induced inflammation (Gaboury *et al*., 1996; Dupuy & Frostell, 1997; Sato *et al*., 1999), general mechanisms which certainly are relevant to pulmonary tissue.

In asthmatic inflammation, the role of NO as an immunomodulator and effector molecule is only beginning to be understood. NOS activity is increased in lung samples of patients with inflammatory lung disease (Belvisi *et al*., 1995) and bronchial epithelial cells show increased immunostaining for NOS in biopsy specimens taken from asthmatic patients in comparison with non-asthmatic controls (Hamid *et al*., 1993). NO suppresses proliferation of Th1 cells, reducing their total excretion of IFN-γ, which favours Th2 proliferation (Taylor-Robinson *et al*., 1994). NO is also involved in the regulation of eosinophil apoptosis (Hebestreit *et al*., 1998). Suppressive effects of NO on mast cell induced inflammation (Gaboury *et al*., 1996) and a decrease of bronchial hyper-responsiveness (Ricciardolo *et al*., 1996; Mehta *et al*., 1997a; Nogami *et al*., 1998; Taylor *et al*., 1998b; Kanazawa *et al*., 1999) have been described.

1.2. BIOLOGICAL MARKERS OF PULMONARY INFLAMMATION

It is now universally accepted that inflammatory processes are central to the pathogenesis of common disease entities such as bronchial asthma, neonatal lung disease and ARDS (Holgate, 1997; Özdemir *et al*., 1997; Artigas *et al*., 1998; Buron *et al*., 1999). The notion that intense and prolonged inflammation is deleterious to lung tissue has resulted in trials of anti-inflammatory treatments in the clinical setting – most commonly the application of glucocorticosteroids inhibiting a broad spectrum of inflammatory mediators (Lukacs & Ward, 1996; Liu & Slutsky, 1997). The demand for individual tailoring of anti-inflammatory therapy calls for an easy, well reproducible and non-invasive technique to monitor ongoing inflammation and therapeutic success. Despite immense research efforts, an ideal tool to that end has not been generally available. Currently used biological markers of pulmonary disease processes are summarised in Tab. 1 and will now be discussed in the context of bronchial asthma. Application of these techniques to the study of neonatal lung disease is only beginning to evolve (Özdemir *et al*., 1997; Viscardi *et al*., 1997; Buron *et al*., 1999).

Pulmonary function parameters

The traditional set to assess disease activity in bronchial asthma and the feature that matters most to the patient is the frequency and degree of clinical symptoms. From a functional point of view, asthma severity is best assessed by studying the degree of airflow limitation. Pulmonary function testing is non-invasive and easy but only an indirect measure of the inflammation that is being treated (Godard *et al*., 1998). Marked obstruction of small airways can escape detection by PEF and FEV1 measurements (Pedersen, 1998). Moreover, airway obstruction may occur over short periods and escape detection by occasional measurements. Even the relationship between bronchial hyperresponsiveness (BHR), the propensity of the airways to contract too much and too easily, and inflammation is insufficient to make standard bronchial challenges with histamine or methacholine reliable indicators of the degree of airway inflammation (Josephs *et al*., 1990; Crimi *et al*., 1998). More recently introduced provocation regimens using indirect stimuli, e.g. exercise, hypertonic saline, or provocation with adenosine-5´-monophosphate (AMP) are similarly impractical for repeated measurement and have only been

Table 1 Evaluation of pulmonary disease processes

SAMPLE	REFERENCE
Pulmonary function parameters	(Holgate, 1998)
Urine	(Dahlén & Kumlin, 1998)
Serum	
markers of eosinophil activity	(Pedersen *et al*., 1996; Kips & Pauwels, 1998)
inflammatory mediators	(Bousquet *et al*., 1998)
lung specific proteins	(Doyle *et al*., 1997; Hermans & Bernard, 1999)
NO metabolites	(Grasemann *et al*., 1997a)
Bronchial biopsies	(Jeffery, 1996; Vignola *et al*., 1998)
Respiratory tract lining fluid	
Bronchoalveolar lavage fluid	
cell counts	(Buron *et al*., 1999)
anti-oxidants	(Kelly *et al*., 1999)
NO metabolites	(Grasemann *et al*., 1997a; Gaston *et al*., 1998)
isoprostanes	(Goil *et al*., 1998; Montuschi *et al*., 1998)
induced sputum	
cytokines	(Kelly *et al*., 1995; Schuster *et al*., 1995; Kips *et al*., 1998)
eosinophil-derived proteins	(Kips *et al*., 1998)
DNA	(Kips *et al*., 1998)
NO metabolites	(Kanazawa *et al*., 1997b)
Breath condensate	
cytokines	(Scheideler *et al*., 1993)
isoprostanes	(Montuschi *et al*., 1999)
NO metabolites	(Ho *et al*., 1998b)
hydrogen peroxide (H_2O_2)	(Sznajder *et al*., 1989; Dohlman *et al*., 1993; Dekhuijzen *et al*., 1996; Antczak *et al*., 1997; Jöbsis *et al*., 1998; Loukides *et al*., 1998a; Worlitzsch *et al*., 1998; Antczak *et al*., 1999)
Exhaled gas	
nitric oxide (NO)	(Gustafsson, 1997 & 1998; **PAPERS I & II**)
carbon monoxide (CO)	(Zayasu *et al*., 1997; Horváth *et al*., 1998; Yamaya *et al*., 1998)
volatile organic compounds (VOC)	(Pitkänen *et al*., 1990; Olopade *et al*., 1997a; Olopade *et al*., 1997b)

used in research settings. Moreover, a possible aggravation of inflammatory activity by the provocation protocol is suggested by a study showing increasing neutrophil influx into sputum during repeated induction with inhaled hypertonic saline (Holz *et al*., 1998).

Serum samples and urine

Given sufficient specificity and sensitivity for pulmonary disease processes, ideal biomarkers for the assessment of disease severity would be those measured in urine (Dahlén & Kumlin, 1998) or blood (Bousquet *et al*., 1998). Studies on urine are only beginning to be performed on a larger scale, although the measurement of leukotriene and prostaglandin metabolites seems promising (Dahlén & Kumlin, 1998). A serological marker, which is increased particularly in asthmatic children (Bousquet *et al*., 1998) and reflects eosinophil activation, is the concentration of eosinophilic cationic protein (ECP) released ex vivo from blood clots (Kips & Pauwels, 1998). Many other circulating mediators, e.g. Th-2 cytokines such as IL-13 or soluble receptors (IL-2R, E-selectin, ICAM-1, α chain of $Fc_{\varepsilon}R1$) show great variability and are therefore not useful in routine clinical practice (Bousquet *et al*., 1998; Holgate, 1998).

Bronchial biopsies

Open lung biopsies to study the degree of asthmatic inflammation are clearly not justified. Therefore, bronchial biopsies are the most direct technique available to assess the nature and magnitude of the pathologic process in asthma (Jeffery, 1998). However, the site of interest may be too peripheral to be reached by bronchial biopsies, let alone the fact that the depth and size of the sample and the admitted frequency of sampling for longitudinal studies limit the use of this technique in routine clinical practice (Jeffery, 1996).

Respiratory tract lining fluid (RTLF)

Available modes to sample the lining fluid of the respiratory tract differ in their respective degree of standardisation. The most standardised yet also the most invasive approach, which traditionally has been used to study the intensity of the inflammatory response mostly in a research setting, is bronchoalveolar lavage (BAL) (Fabbri *et al*., 1998). Both BAL and bronchial washes, a somewhat less standardised approach, are invasive in that they require endotracheal intubation or bronchoscopy (Scheinmann *et al*., 1998). A less invasive mode of sampling RTLF, the collection of induced sputum, does not require instrumental access to the trachea. It has recently become more standardised, making this non-invasive approach useful even in the investigation of paediatric patients (Holgate, 1998; Kips *et al*., 1998; Scheinmann *et al*., 1998). Induced sputum samples obtained by a standardised protocol

and investigated for their cellular content and soluble mediators such as proinflammatory cytokines and neutrophil elastase can be useful to assess inflammatory activity in bronchial asthma (Kips *et al*., 1998). Possible salivary contribution to the levels of cytokines (Jöbsis *et al*., 1998) and NO metabolites (Zetterquist *et al*., 1999) in the sputum sample necessitates correction for different degrees of salivary contamination (Kips *et al*., 1998).

Breath condensate

Collection of breath condensate, another non-invasive approach to study ongoing inflammatory processes in the lung, can be performed in spontaneously breathing subjects and, with some more technical sophistication, in intubated individuals (Baldwin *et al*., 1986; Kietzmann *et al*., 1993). Activation of the inflammatory cascade in the lung leads to recruitment of macrophages and neutrophils and increased generation of oxygen free radicals, including hydrogen peroxide (H_2O_2) (Greening & Lowrie, 1983; Winterbourn, 1996; Antczak *et al*., 1999). The latter can be detected in the exhalate of spontaneously breathing adult and paediatric patients with bronchial asthma (Dohlman *et al*., 1993; Antczak *et al*., 1997; Jöbsis *et al*., 1998; Antczak *et al*., 1999). In addition to H_2O_2, cytokines can be detected in breath condensate of spontaneously breathing subjects (Scheideler *et al*., 1993). As is the case with sputum samples (see above), saliva contamination of breath condensate cytokines needs to be excluded. Another group of bioactive molecules, which can be studied in tracheal aspirates or breath condensate and which do not occur in saliva are isoprostanes. These peroxidation products of prostaglandin metabolism are increasingly formed during oxidative stress (Morrow & Roberts, 1997). They may have biological effects in the respiratory tract (Hill *et al*., 1997; Bernareggi *et al*., 1998). One study has reported increased concentrations of an F_2 isoprostane in breath condensate of asthmatic patients and a positive correlation to exhaled NO in patients with mild asthma (Montuschi *et al*., 1999).

Exhaled gas analysis

In a sample of breath more than 250 substances can be differentiated using gas chromatography (Pauling *et al*., 1971; Teranishi *et al*., 1972), but interest in using exhaled gas in the investigation of pulmonary disease processes has occurred relatively lately. Apart from NO, which is further discussed below, carbon monoxide (CO) and volatile organic compounds (VOC) are now being studied on a larger scale. Measurements of exhaled CO have in the past been used to evaluate smoking habits (Jarvis *et al*., 1980). Endogenous CO can arise from the metabolism of haeme by haeme oxygenase 1 (HMOX1, EC 1.12.99.3) (McKusick, 1988). In case of increased oxidative stress (Otterbein *et al*.,

1995) and/or stimulation by pro-inflammatory cytokines (Cantoni *et al*., 1991) HMOX1 is induced in airway macrophages (Horváth *et al*., 1998) and increased levels of CO can be detected in exhaled gas (Zayasu *et al*., 1997; Horváth *et al*., 1998). Similar increases in exhaled CO have been detected in viral upper respiratory tract infection (Yamaya *et al*., 1998). HMOX1 is up-regulated by NO (Kim *et al*., 1995), casting some doubt on the role of exhaled CO as an inflammatory marker independent of NO. The existence of VOC has been known for a long time but was initially ascribed to peculiarities of the diet and whole body metabolism (Jansson & Larsson, 1969). It was then realised that the process of free radical formation and consecutive peroxidation of polyunsaturated fatty acids, i.e. of linoleic and linolenic acid, induced the pulmonary formation of volatile hydrocarbons (ethane and pentane) which could be quantified in breath samples (Massias *et al*., 1993). These findings are only beginning to be applied to the study of bronchial asthma (Kharitonov & Barnes, 1996). Increased pentane levels in exhaled gas are not specific for pulmonary inflammation but are also seen in inflammatory bowel disease (Kokoszka *et al*., 1993) and after myocardial infarction (Weitz *et al*., 1991).

NO in exhaled gas

In 1991 Gustafsson and co-workers reported that NO was present in the exhaled gas of rabbits guinea-pigs and humans and was endogenously produced in the lung (Gustafsson *et al*., 1991). This finding has since been confirmed in a large number of mammalian species (Schedin *et al*., 1995a; Lewandowski *et al*., 1996; Gustafsson, 1997; Schedin *et al*., 1997b; Lewandowski *et al*., 1998). Studying NO exhaled from the lower airways offers a unique possibility to study features of pulmonary NO metabolism non-invasively (Gustafsson, 1997). This is particularly true for asthmatic inflammation, which goes along with increased levels of NO in the exhaled gas of adults and children suffering from bronchial asthma (Alving *et al*., 1993b; Kharitonov *et al*., 1994b; Persson *et al*., 1994c; Dötsch *et al*., 1996; Lundberg *et al*., 1996a; **PAPER I**). However, the anatomic site and main cell type producing the NO molecules detected in exhaled gas are still a matter of debate. Corollaries to this are the open questions how pulmonary tissue concentrations of NO and the various known biological functions of NO (chapter 1.1) are reflected in exhaled gas.

More than 90% of exhaled NO may under certain conditions derive from the upper airways (Alving *et al*., 1993b; Lundberg *et al*., 1994a; Lundberg *et al*., 1994c) and the concentrations are particularly high in the paranasal sinuses (Lundberg *et al*., 1994a; Lundberg *et al*., 1995a; Rinder, 1996). The fact that the instillation of a NOS inhibitor directly into the sinus lumen is much more potent in reducing nasal NO concentrations than the inhalation of aerosolised NOS inhibitor suggests that most of the NO found in nasal gas reached the nasal cavity via diffusion from the paranasal sinuses (Lundberg *et al*., 1994a; Lundberg *et al*., 1995a). This notion is supported by in situ mRNA hybridisation with human hepatocyte iNOS and immunostaining for mouse macrophage iNOS which

labelled epithelial cells in the sinus whereas immunostaining was only weak and patchy in the nasal epithelium (Lundberg *et al*., 1995a). While some nasal NO is locally absorbed (Dubois *et al*., 1998), the major portion of nasally derived NO leaves the nasal cavity with the respiratory gas stream, i.e. it is exhaled or autoinhaled. The idea that autoinhalation of NO produced in the upper respiratory tract might influence ventilation perfusion matching in the lung was first proposed by Gerlach and co-workers (Gerlach *et al*., 1994). It has since been shown that adding nasal gas to the air inspired by long-term intubated patients at a final NO concentration of 20 - 40 ppb results in significant increases of arterial P_aO_2 (Lundberg *et al*., 1995b; Lundberg *et al*., 1996b). Oxygenation as indicated by transcutaneous oxygen tension ($tcPO_2$) also improves in some healthy individuals during nasal breathing and thus autoinhalation of nasally-produced NO (Lundberg *et al*., 1996b). Nasal breathing has also been demonstrated to lower pulmonary vascular resistance index in comparison with oral breathing in a group of patients after open heart surgery (Settergren *et al*., 1998). Residual NO in hospital compressed air (2-550 ppb) used to ventilate patients with healthy lungs postoperatively has been shown to improve oxygenation (Lee *et al*., 1997). These concentrations are in the range of ambient NO concentrations and of those found in the upper respiratory tract of healthy neonates (**PAPER III**).

Notwithstanding the clear evidence that NO in exhaled gas can mainly originate from enzymatic NO synthesis in the paranasal sinuses, significant amounts of NO are exhaled from the lower airways (Gustafsson, 1997). In healthy humans NO can be detected in end-expiratory, i.e. alveolar gas (Borland *et al*., 1993; Persson *et al*., 1993b) and by direct sampling from the trachea via endotracheal tubes or during bronchoscopy (Gerlach *et al*., 1994; Schedin *et al*., 1995b; Dillon *et al*., 1996; Kharitonov *et al*., 1996a; Massaro *et al*., 1996; Gabbay *et al*., 1998). On the cellular level the major contributor to the total amount of NO exhaled from the lower airways is most probably the bronchial epithelial cell for several reasons. Firstly, due to the enormous capacity of circulating haemoglobin to scavenge NO molecules (Rimar & Gillis, 1993), it is doubtful whether NO produced in the interstitium will reach the exhalate in the *in vivo* situation (Persson *et al*., 1994b; Cremona *et al*., 1995; Gustafsson, 1997). Secondly bronchial epithelial cells are the most abundant cell type at those sites in the lower airways where most NO formation is suspected. A predominant source within the tracheobronchial tree is suggested by synchronised single exhalation profiles of NO and CO_2. NO peaks when CO_2 begins to reach its end-expiratory plateau but then declines towards the very end of exhalation (Persson *et al*., 1993b). Moreover, the mean times to reach peak levels are significantly shorter for NO compared with CO_2 (Byrnes *et al*., 1997). This suggests formation mainly in the terminal and respiratory bronchioles (Persson *et al*., 1993b). Later studies have supported this interpretation by showing that dead space for NO is ca. 50 % smaller than for CO_2 (Tsoukias *et al*., 1998) and that breathholding increases NO concentration mainly in the initial 200 ml of gas exhaled by adult intubated patients (Tsujino *et al*., 1996). A significant production of NO contributing to total

exhaled NO has been found in the large airways including the trachea (Silkoff *et al*., 1998b; DuBois *et al*., 1999). In a recent study, intravenous application of the NOS inhibitor L-NMMA caused clear haemodynamic effects but did not evoke significant changes of exhaled NO concentrations, whereas inhalation of aerosolised L-NMMA dramatically decreased exhaled NO levels without causing haemodynamic changes (Sartori *et al*., 1999). This again suggests that the source of NO production contributing to exhaled NO is located very superficially in the airways and almost certainly not in the vessels of the pulmonary circulation. Letting alone changes of respiratory gas flow, which are discussed below (chapter 3.3), excretion of NO into exhaled gas will be determined by the rate of production and the rate of diffusion into the surrounding pulmonary tissues including blood vessels (Hyde *et al*., 1997; DuBois *et al*., 1999; Geigel *et al*., 1999). Factors regulating the total amount of NO found in exhaled gas will be discussed below (chapter 3.3).

All isoforms of NOS are constitutively expressed in lung tissue. Endothelial NOS can be found in pulmonary and bronchial arteries and veins showing maturational changes of immunoreactivity with a peak immediately after birth in pigs (Hislop *et al*., 1995) and similar developmental regulation in rats (Kawai *et al*., 1995). Neuronal NOS can be found in nerve fibers in airway smooth muscle, submucosal glands and blood vessels as well as in airway intrinsic ganglia (Fischer & Hoffmann, 1996). Others have shown that iNOS is constitutively expressed in human airway epithelium (Guo *et al*., 1995). This constitutive expression seems to be dependent on conditions and/or factors within the airway, because it is rapidly lost on removal of the cells from the in vivo environment (Guo *et al*., 1995). In isolated perfused rabbit lungs the presence of NO in the exhaled gas is partly sensitive to the concentration of calcium in lung perfusate (Persson *et al*., 1994b).

1.3. BRONCHIAL ASTHMA IN CHILDREN

> *Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, including mast cells and eosinophils. In susceptible individuals this inflammation causes symptoms which are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment, and causes an associated increase in airway responsiveness to a variety of stimuli. (National Heart, Blood and Lung Institute, 1992)*

Childhood asthma is one of the most common diseases encountered by the paediatrician. Over the last 30 years the prevalence of childhood asthma has been increasing in many countries (Pedersen, 1998). According to conservative estimates bronchial asthma affects 10 % of children in western countries (Burney *et al*., 1990). This secular trend remains unexplained and is subject to intensive research. While childhood and adult asthma share the same underlying pathopysiological mechanisms there are some important anatomic and physiological differences which justify investigations into diagnostic tools and therapeutic options of their own right (Pedersen, 1998). For example, the reliable and accurate assessment of the clinical condition presents particular problems in children (Pedersen, 1998). It is well known that an adequate assessment of symptom control by history taking (**PAPER II**) requires careful education of the children and their parents as well as time, skill and experience on the physician's side (Pedersen, 1998). These pre-requisites which are at best available in specialised outpatient departments (**PAPERS I & II;** Doerschug *et al*., 1999). It has already been said that assessment of the clinical status using pulmonary function testing is likewise problematic (chapter 1.2.). Having said that, history-taking and assessment of airflow obstruction still are the classical tools to assess asthma severity, so that a new diagnostic option such as the measurement of exhaled NO has to be compared with them. Current asthma therapy is based on the assumption that there is a close relationship between clinical asthma severity and airways inflammation. Assuming that inflammatory activity is worst in those children with the most intense or most recent clinical symptoms, any useful biomarker of pulmonary inflammation should reflect this.

2. AIMS OF THE STUDY

When this project was begun in early 1994, only a small number of papers on the topic of NO in the exhaled gas had been published (Gustafsson *et al*., 1991; Alving *et al*., 1993b; Persson *et al*., 1993a; Persson & Gustafsson, 1993; Persson *et al*., 1993b; Gerlach *et al*., 1994; Kharitonov *et al*., 1994b; Persson *et al*., 1994c). Nonetheless it was becoming clear that exhaled NO measurement was a promising new diagnostic option to assess ongoing cytokine-induced pulmonary inflammation in adult patients (Alving *et al*., 1993b; Kharitonov *et al*., 1994b; Persson *et al*., 1994c).

The main objectives of this study were

- to assess the applicability of exhaled NO measurements in children and neonates (**PAPERS I-III**),
- to optimise existing sampling techniques (**PAPER II**) and adapt them to the specific needs of children and infants (**PAPER II & III**),
- to assess a possible difference of exhaled NO between children suffering from asthma and healthy controls (**PAPERS I & II**),
- to study a possible link between exhaled NO analysis and clinical severity in asthmatic children (**PAPER II**),
- to study airway NO concentrations during tidal breathing in neonates (**PAPER III**),
- to study regulatory aspects of pulmonary NO production measuring exhaled NO in an established rabbit model (**PAPERS IV & V**).

3. MATERIALS AND METHODS

This chapter mainly covers general methodological considerations. For a detailed account of materials and methods the reader is referred to the individual papers.

3.1. PATIENTS

Children with bronchial asthma

As mentioned earlier (chapter 1.3.) a clinically useful biomarker of airway inflammation should be normal in healthy individuals, altered in those with ongoing inflammation, who are still symptom-free, and even more deranged in those subjects with clinical symptoms arising from a more intense inflammation. **PAPER II** investigated whether exhaled NO measurements might reflect this continuum of inflammatory activity. In this study, the severity of asthma in children was classified into clinical patterns according to current definitions. In **PAPER I** a subclassification into mild, moderate and severe asthma on the basis of the 1992 statement of the International paediatric asthma consensus group was employed (1992), whereas **PAPER II** used the terminology of the present consensus (Warner & Naspitz, 1998). The introduction of the new consensus statement (Warner & Naspitz, 1998) also explains the fact that the asthmatic children in **PAPER II** inhaled disodium chromoglycate in addition to glucocorticosteroids. This option was found in the previous guidelines (International paediatric asthma consensus group, 1992) but eliminated from the new edition (Warner & Naspitz, 1998).

Newborn infants

NO can be detected in the upper airways of newborns immediately after birth (Schedin *et al*., 1996). This raises the question (Schedin *et al*., 1996) whether the paranasal sinuses are sufficiently aerated in this age group to account for NO production just as they do in adults (cf. chapter 1.2). The paranasal sinuses in newborns, mainly the maxillary and ethmoid sinuses, are of considerable size (Parsons-Schaeffer, 1920; Graney, 1986; Wolf *et al*., 1993). Their patency is clinically revealed by the occasional occurrence of sinusitis even in infancy (Kendall & Senders, 1996). However, radiological investigations have revealed a high rate of maxillary opacification in asymptomatic children under the age of 2 years (Odita *et al*., 1986; Glasier *et al*., 1989). Effects of those incidental sinus abnormalities on the availability of nasal NO have not been studied. In analogy to adults with sinusitis or non-allergic polyps a decrease of available nasal NO is conceivable (Baraldi *et al*., 1997; Arnal *et al*., 1999), but has not been investigated. NO release from the well-developed ethmoid sinuses (Graney, 1986; Wolf *et al*., 1993) might compensate for any shortage of maxillary NO. Summing up, there is at present no

convincing evidence to suggest that the origin of upper airway NO in neonates should be any different from adult subjects.

3.2. THE RABBIT MODEL

NO exhaled from the rabbit lung has been studied by various groups for a number of years both in the intact animal (Persson *et al*., 1990; Gustafsson *et al*., 1991; Sprague *et al*., 1992; Högman *et al*., 1994; Carlin *et al*., 1997b) and in isolated perfused lung (Persson *et al*., 1994b; Grimminger *et al*., 1995; Carlin *et al*., 1997a; Adding *et al*., 1999). The rabbit is an attractive species to study because, in contrast to many other species and the human (Schedin *et al*., 1995b; Gustafsson, 1997), rather high concentrations of NO (e.g. 17 ppb ; Persson *et al*., 1994b) can be detected in mixed exhaled gas from rabbit trachea. Moreover, NO produced in the lower airways and lung does not interfere with relatively high concentrations of NO from the nose, as can be found in the human and in many other mammalian species (Lundberg *et al*., 1994c; Schedin *et al*., 1995b; Lewandowski *et al*., 1996; Mills *et al*., 1996; Schedin *et al*., 1997b). It has recently been shown that even single breath analysis of exhaled NO is reliable in this animal model in spite of small tidal volumes and high respiratory rates (Adding *et al*., 1999).

Sodium pentobarbital has traditionally been used in our group to induce and maintain anaesthesia in rabbits and was even used in the present experiments **(PAPER IV & V)**. There is a large body of experimental data on exhaled NO analysis in the pentobarbital anaesthetised rabbit. It was felt that this should be given priority over the known fact that pentobarbital clearly has a more suppressive effect on the autonomic nervous system than inhalational anaesthetics such as isoflurane (Duan *et al*., 1994). To differentiate between β_1- and β_2-mediated effects on pulmonary NO production (**PAPER IV**) it was decided to compare the effect of prenalterol and terbutaline infusions. It has been argued that prenalterol is not a pure β_1-adrenoceptor agonist (Apperley *et al*., 1982). However, random order of prenalterol and terbutaline infusions and the clear difference between the respective effects on exhaled NO concentration strongly support the central finding of our work that the stimulatory effect on NO production is mediated by β_1- adrenoceptors. Nimodipine as a Ca^{2+}-channel blocking agent was chosen because, being a dihydropyridine, it blocks the well-characterised L-subtype of voltage-gated Ca^{2+}-channels which had previously been shown to be directly coupled to activated G-proteins (Brown & Birnbaumer, 1988). Moreover, nimodipine has only little effect on cardiac contractility, automaticity and conduction (Robertson & Robertson, 1996).

3.3. NO ANALYSIS

Technique of sampling

In the first study (**PAPER I**) exhaled NO was measured in mixed exhaled gas during oral breathing while wearing a nose clamp. Unfortunately, our measurements in orally exhaled gas suggested some degree of contamination with nasally derived NO (**PAPER I**), something that was confirmed by later study in adults (Kimberly *et al*., 1996; Silkoff *et al*., 1997). Therefore an optimised separation of nasal and lower airway NO by application of a continuous nasal suction was sought (**PAPER II**). At the same time the European Respiratory Society recommended NO measurements during single slow exhalations against a flow resistance (Kharitonov *et al*., 1997). The ensuing increase of pharyngeal pressure closes off the soft palate thus minimising nasal contribution to NO measurements in oral gas (Kharitonov *et al*., 1996a; Kharitonov & Barnes, 1997; Silkoff *et al*., 1997). In adults these measurements show an excellent correlation with those using the nasal suction technique (R = 0.99, T. Busch, Berlin, unpublished observation). Another factor complicating the measurement of exhaled lower airway NO is its dependency on expiratory flow. There is a clear and non-linear decrease of exhaled NO concentration and increase of NO excretion between expiratory flows of 50 and 300 ml s^{-1} (Högman *et al*., 1997; Silkoff *et al*., 1997). To compensate for this, constant exhalation flow rates have hence been recommended for exhaled NO measurements (Kharitonov *et al*., 1997). This is usually performed using a pneumotachograph connected to a visual analogue scale so that the subject can regulate his or her expiratory flow accordingly. An own recent study however raises considerable doubt about the ability of children under the age of 10 years to stabilise their expiratory flow sufficiently (manuscript in preparation).

Lacking co-operation of small infants, their natural tendency to breathe through the nose (Rodenstein & Stanescu, 1986; Shatz *et al*., 1994) and the need for maximum non-invasiveness required the development of a new mode of sampling for newborns (**PAPER III**). Single breath measurements in gas sampled from the pharynx also allowed the measurement of autoinhaled nasal NO. The measurements were done in the absence of any effects from nasal decongestants. This is important to state because topically applied α-adrenoceptor agonists cause an acute decrease of nasal cavity NO levels (Rinder, 1996; Rinder *et al*., 1996; Ferguson & Eccles, 1997). Another effect of topical vasoconstrictor application to the nasal mucosa is the interruption of the nasal cycle (Zinreich *et al*., 1988; Eccles, 1996), which is further discussed in the context of our observations in neonates (**PAPER III**).

Technique of measurement

The presence of NO in exhaled gas has been proven using various detection techniques including chemiluminescence, diazotisation assays and chemical trapping followed by gas chromatography-mass spectrometry (GC-MS) (Gustafsson *et al*., 1991), direct GC-MS (Leone *et al*., 1994) and cold trap experiments (Persson & Gustafsson, 1993; Persson *et al*., 1994b). Due to their high sensitivity, chemiluminescence detectors (CLD) are now universally used to detect exhaled NO (**PAPERS I-V**). Briefly, it is based on the propensity of NO molecules to generate light in a chemical reaction with ozone, which takes place under low pressure in a reaction chamber. A photomultiplier tube detects the emitted photons converting the physicochemical signal to an electric impulse, which can be processed by a complicated array of electronic amplifiers. Unfortunately the wavelength of the emitted photons is in the range > 600 nm (Zafiriou & McFarland, 1980; Body & Hartigan, 1997), whereas the maximum sensitivity of even the best photomultiplier tubes is at shorter wavelengths (300 - 600 nm - manufacturers information). There is an intricate balance between sampling flow, intensity of the primary signal, vacuum in the reaction chamber, ozone supply, and the signal to noise ratio of the CLD. The latter is determined by the physical characteristics of the tube and the quality, set-up and temperature of the amplifier circuit. This is the reason for individual differences of sensitivity between CLDs and necessitates specification of the detection level for any CLD used (**PAPERS I-V**). The CLD has a linear response up to 1 ppm (manufacturer's information). Recent studies suggest that water vapour may quench the chemiluminescence signal substantially (Kharitonov *et al*., 1997; van der Mark *et al*., 1997). This systematic error, which does not affect the significance of the results of **PAPERS I & II**, was in the second part of this study minimised by interconnected dehumidifying tubes in the sampling tubes (**PAPERS III-V**). The quenching effect of CO_2 in the 0-10% range on the chemiluminescence signal is ≤ 10% (Strömberg *et al*., 1997; van der Mark *et al*., 1997).

3.4. HIGH FREQUENCY OSCILLATORY VENTILATION (HFOV)

HFOV is a mode of artificial ventilation the physical characteristics of which can be characterised by small "tidal" amplitudes applied at very high rates (Greenough, 1994). Due to its proven clinical benefit, HFOV is now widely used to alleviate common neonatal pulmonary diseases (Greenough, 1994; Ring & Stidham, 1994; Gerstmann *et al*., 1996). In contrast to the diseased lungs of these newborns, we used HFOV to ventilate rabbits with healthy lungs (**PAPER V**) in order not to confound the measurements with NO induced by the inflammatory process of any model of lung disease. HFOV respirators in general use various technical devices to generate oscillating ventilation patterns, including pistons, moving gas rapidly back and forth in the breathing circuit (e.g. Stefan SHF 3000®, Hummingbird V®, Dufour OHF1®), large loudspeaker membranes (Sensormedics 3100 A®) and expiratory jet Venturi systems in combination with a high continuous inspiratory gas flow (Dräger

Babylog 8000®, **PAPER V**). These differences in construction notwithstanding, all these oscillators provide active "inspiration" and active "expiration" with more or less sinusoidal pressure waveforms (Stachow, 1995). Irrespective of the brand of the respirator used, the process of HFOV is determined by three parameters, namely the oscillatory volume resulting from these pressure swings, the mean airway pressure, around which the pressure oscillates, and the oscillatory frequency.

4. RESULTS AND DISCUSSION

4.1. EXHALED NO IN CHILDHOOD ASTHMA (PAPERS I & II)

The main findings of these papers are that

- exhaled NO can be measured in the paediatric age group.
- contamination with nasally produced NO can be minimised.
- exhaled NO is significantly increased in asthmatic as compared with healthy children.
- exhaled NO concentrations and markers of airflow obstruction do not correlate.
- exhaled NO is significantly increased in asthmatic children with recent clinical symptoms.

A correlation of exhaled NO measurements with asthma severity has long been sought for several reasons. Firstly, as was already outlined above (chapter 1.3.), the usefulness of any biomarker of asthmatic inflammation depends on a close correlation with the functional changes in the respiratory system, e.g. symptoms, degree of airflow obstruction or altered bronchial hyper-responsiveness. Secondly, experimental evidence has suggested links between exhaled NO and these functional changes. Following experimental antigen challenge an immediate but short-lasting increase of exhaled NO has been reported in ovalbumin-sensitised guinea pigs (Persson *et al*., 1993a). Already a few minutes after direct (allergen) or indirect stimuli (histamine, 5-AMP or isocapnic cold air hyperventilation), exhaled NO decreases to levels at or below baseline while airflow remains to be obstructed (Persson & Gustafsson, 1993; Kharitonov *et al*., 1995b; de Gouw *et al*., 1998b; Deykin *et al*., 1998; Therminarias *et al*., 1998b; Paredi *et al*., 1999). In patients showing an early and late airway obstruction following the initial stimulus (dual responders), exhaled NO and bronchial tone increase significantly 6-10 h after the challenge (Kharitonov *et al*., 1995b; Paredi *et al*., 1999). Opposite changes of exhaled NO during the early and late response to exogenous stimuli may explain the fact that clinical studies trying to correlate exhaled NO and clinical symptoms, pulmonary function parameters, or bronchial hyperreactivity, have been conflicting. In accordance with our study (**PAPER II**), Kharitonov and co-workers found a decrease of nocturnal symptoms, diurnal peak expiratory flow variability and airway-hyperresponsiveness with decreasing exhaled NO concentrations in adult asthmatic patients (Kharitonov *et al*., 1996b; Kharitonov *et al*., 1996c). Others did not find a link

between exhaled NO and nocturnal airway obstruction (ten Hacken *et al*., 1998), airflow obstruction or bronchial hyperresponsiveness (ten Hacken *et al*., 1996). A weak and moderately significant negative correlation between exhaled NO and FEV1 has only occasionally been reported (Lim *et al*., 1999; **PAPER I**). While overwhelming evidence dismisses a close link between parameters of airflow obstruction and exhaled NO (Kharitonov *et al*., 1996b; Kharitonov *et al*., 1996c; ten Hacken *et al*., 1996; al-Ali *et al*., 1998; Stirling *et al*., 1998; **PAPER II**), a weak negative correlation is to be expected due to the relation between expiratory flow and exhaled NO.

Our finding that recently symptomatic children treated with inhaled glucocorticosteroids (n = 5) had higher NO excretion rates than their symptomatic steroid-naïve counterparts (n = 8) (**Fig. 2 in PAPER II**) was not statistically significant. However, it is paralleled by a similar observation in a larger group of adult patients with difficult asthma (n = 26) reaching statistical significance ($p < 0.05$; Stirling *et al*., 1998). While lack of compliance with the prescribed therapy might potentially explain the findings in our study (**PAPER II**), Stirling and co-workers could make this unlikely using serum prednisolone measurements (Stirling *et al*., 1998). The observation is probably explained by the fact that those symptomatic individuals with more severe disease receive more intensive treatment. Alternatively, subjects with symptoms in spite of intensive anti-inflammatory therapy may represent a subgroup of asthmatic patients in whom NO levels are unaffected or only marginally decreased by glucocorticosteroids (Stirling *et al*., 1998).

In spite of clear-cut differences in exhaled NO levels and excretion rates, our measurements did not reveal significant differences of pulmonary function test results between symptom-free children and those with recent symptoms. This included insignificant differences in peak expiratory flow rate (PEF) (results not shown). Just as lacking correlation between exhaled NO and airflow obstruction in the group of asthmatic patients as a whole, this raises the question which biomarker of disease activity is the most useful for the management of the patients. Assessment of clinical symptoms is essential but not perfect and should be accompanied by other methods to assess airway inflammation (Godard *et al*., 1998). It has already been pointed out that pulmonary function tests and the measurement of bronchial hyperresponsiveness do not show a perfect connection to the intensity of asthmatic inflammation (chapter 1.2.). On the other hand pulmonary NO production is linked to the process of asthmatic inflammation (cf. chapter 1.1.) and exhaled NO levels are related to airway hyperresponsiveness (al-Ali *et al*., 1998; Dupont *et al*., 1998; Salome *et al*., 1999), blood eosinophilia (Salome *et al*., 1999; Silvestri *et al*., 1999), sputum eosinophil markers (Mattes *et al*., 1999; Piacentini *et al*., 1999) and urinary markers of eosinophilic activation (Mattes *et al*., 1999). Moreover, superior sensitivity of exhaled NO measurement over serum markers of asthmatic inflammation has been claimed (Lanz *et al*., 1997). Given the link between exhaled NO and disease severity (Stirling *et al*., 1998; **PAPER II**) and the correlation of exhaled NO with clinical

improvements during glucocorticosteroid therapy as well as with glucocorticosteroid dose (Massaro *et al*., 1995; Kharitonov *et al*., 1996b; Kharitonov *et al*., 1996c; Lundberg *et al*., 1996a; Lim *et al*., 1999), we suggest that exhaled NO measurements in bronchial asthma may at least be a useful adjunct to pulmonary function testing and may help to monitor the effectiveness of anti-inflammatory therapy even in the paediatric age-group.

Table 2 Factors influencing exhaled NO in humans

FACTOR	REFERENCE
Increases of exhaled NO	
L-arginine ingestion	(Kharitonov *et al*., 1995a)
inhalation of L-arginine	(Sapienza *et al*., 1998)
alveolar hypoxia	(Dweik *et al*., 1998)
acute cigarette smoke inhalation	(Chambers *et al*., 1998)
breathholding	(Persson *et al*., 1994c; Kharitonov *et al*., 1996a; Kimberly *et al*., 1996; Tsujino *et al*., 1996; Silkoff *et al*., 1997)
physical exercise (increases NO excretion only)	(Persson *et al*., 1993b; Bauer *et al*., 1994; Iwamoto *et al*., 1994; Trolin *et al*., 1994; Phillips *et al*., 1996)
contribution from saliva & diet	(Zetterquist *et al*., 1999) (Silkoff *et al*., 1998b)
regurgitation from stomach	(Lundberg *et al*., 1994b)
voluntary hyperventilation (increases NO excretion only)	(Persson *et al*., 1993b)
mid menstrual cycle	(Kharitonov *et al*., 1994a)
upper respiratory tract infection	(Alving *et al*., 1993b; Kharitonov *et al*., 1995e; de Gouw *et al*., 1998a)
Only in asthmatic patients: β_2–agonist inhalation	(Ho *et al*., 1997; Yates *et al*., 1997; Silkoff *et al*., 1999) no change: (Garnier *et al*., 1996)
Decreases of exhaled NO	
chronic cigarette smoking	(Gerlach *et al*., 1994; Persson *et al*., 1994c; Schilling *et al*., 1994; Kharitonov *et al*., 1995c; Robbins *et al*., 1996)
acute alcohol ingestion	(Persson *et al*., 1994a; Yates *et al*., 1996)
alcoholic disinfectants	(Meijer *et al*., 1996)
core body cooling	(Pendergast *et al*., 1999)

4.2. TIDAL NO MEASUREMENTS IN NEONATES (PAPER III)

The main findings of this paper are that

- tidal NO analysis can differentiate inhaled and exhaled NO concentration in the newborn.
- exhaled NO during regular breathing can be measured with good repeatability.
- inhaled NO from the upper airways reaches concentrations which likely have biological effects in the lower respiratory tract.
- transient decreases of inhaled NO concentrations might have physiological significance.

Upper airway NO in the newborn is probably produced mainly in the paranasal sinuses just as it is in adults (cf. chapter 3.1.). As the volume of aerated paranasal sinuses and hence the epithelial surface area is considerably smaller at birth compared with later ages (Parsons-Schaeffer, 1920; Graney, 1986), a lower rate of NO production should ensue. Indeed, NO excretion during spontaneous breathing is considerably lower in newborns in comparison with older children and adults (Artlich *et al*., 1998). Relating NO excretions to body weight yields comparable rates of NO release per kg body weight across all ages of air breathing (Schedin *et al*., 1995b; Kimberly *et al*., 1996; Schedin *et al*., 1996; Schedin *et al*., 1997a; Artlich *et al*., 1998).

The fate of autoinhaled NO (e.g. 52.2 ± 5.8 ppb, **PAPER III**) in the lower respiratory tract must be considered because residuals of NO in the dead space might potentially confound the measurement of NO concentration during the consecutive exhalation. Due to the immense capacity of the respiratory system to absorb and inactivate NO, which is not restricted to the alveoli but also occurs in the trachea and bronchi (DuBois *et al*., 1999), confounding effects between breaths is unlikely. For example, inhalation of NO calibration gas (113-800 ppb) does not affect the peak exhaled NO in normal subjects (Kharitonov *et al*., 1994b; Robbins *et al*., 1996). Higher NO concentrations in the first 200 ml of exhaled gas compared to the second fraction have been reported (Tsujino *et al*., 1996). This was however also seen in intubated patients, suggesting a tracheobronchial source of NO production rather than the presence of residual NO from the preceding inhalation. This conclusion does not contrast with recent findings that exogenous NO (3-430 ppb) can influence exhaled NO measurements (Baraldi *et al*., 1998; Deykin *et al*., 1998; Therminarias *et al*., 1998a), because the increases seen in those studies are of endogenous origin (Steerenberg *et al*., 1999). In summary, current evidence does not indicate that residual NO from the preceding inhalation confounds NO measurements in the exhaled gas.

4.3. ADRENALINE AND PULMONARY NO PRODUCTION (PAPER IV)

The main findings of this paper are that

- pulmonary NO production can be stimulated via adrenoceptors in anaesthetised rabbits.
- the stimulatory effect on NO production is mediated by β-adrenoceptors.
- stimulation with β_1-selective agonists is more potent than with β_2-selective agonists.
- the stimulatory effect of β-adrenoceptor agonists is also seen in blood-free buffer-perfused lungs under conditions of constant flow.

Current evidence argues strongly against the theoretical possibility that a mere change pulmonary blood flow might account for the observed increase of NO levels in response to adrenoceptor stimulation (**PAPER IV**; Mehta *et al*., 1997b; Pogliaghi *et al*., 1997). Only extreme hypovolaemia has been shown to lead to increases in exhaled NO concentration (Carlin *et al*., 1997b). Our finding that β-adrenoceptor stimulation can lead to increased pulmonary NO production in the lung is compatible with recent reports that the inhalation of β_2 agonists can evoke transient increases of exhaled NO concentrations in patients with asthma (Ho *et al*., 1997; Yates *et al*., 1997; Silkoff *et al*., 1999). Even so, it is important to keep in mind that our study involves rabbits and that there may be species variation in the relative proportion of β_1 and β_2 adrenoceptors. Coexistence of β_1 and β_2 adrenoceptors has been demonstrated in both human and rabbit lungs (Rugg *et al*., 1978; Carstairs *et al*., 1985). However, a predominant expression of β_1 adrenoceptors in rabbits and of β_2 adrenoceptors in human lungs has been claimed (Mak *et al*., 1996). Therefore some caution transferring results from rabbits to the human is warranted. In the human, β-adrenoceptors regulate many aspects of airway function, are widely distributed in human lungs and have been localised to many cell types (Carstairs *et al*., 1985). Both the β_1- and the β_2-subclass of receptors have been identified in vascular smooth muscle, airway epithelium and alveolar type II cells (Davis & Kercscmar, 1991; Isohama *et al*., 1995; Mak *et al*., 1996). Other cellular components of the lungs and airways, e.g. respiratory smooth muscle, submucosal glands and Clara cells, contain abundant β adrenoceptors mainly of the β_2 variety (Davis & Kercscmar, 1991). Finally, pulmonary mast cells, the mucosal subpopulation of which is often seen in close association with unmyelinated nerve endings, express β-adrenoceptors and have been functionally linked to suppressed histamine release after allergen exposure (Assem & Schild, 1973). Mechanical removal of airway epithelium reduces the bronchodilator effect of β-agonists, suggesting that β-agonists may release a relaxing factor from airway epithelial cells (Barnes *et al*., 1985; Flavahan *et al*., 1985).

According to our results, the intracellular mechanisms involved in mediating the effect of β-adrenoceptors on pulmonary NO production do not seem to involve the generation of cAMP as a second messenger, as is usually the case (Hoffman *et al*., 1995). Interestingly, it has recently been shown that relaxation of airway smooth muscle in response β-agonists can occur via a direct effect of G-protein on Ca^{2+}-activated potassium (K_{Ca}) channels and independently of a rise in intracellular

cAMP (Kume *et al*., 1994; Barnes, 1997). In heart and skeletal muscle there is moreover evidence for the existence of β_1-adrenoceptors with direct coupling to calcium channels (Brown & Birnbaumer, 1988). Our finding that the effect of adrenoceptor stimulation decreases with inhibition of L-type calcium channels by nimodipine hints at the existence of an analogous mechanism in the NO-producing cells in the lung.

Plasma adrenaline concentrations following adrenaline infusion were not measured in this study. However, more recent work in rabbits has shown that adrenaline infusions (3 $\mu g\ kg^{-1}\ min^{-1}$) results in plasma concentrations in the order of 25 nM (Adding, 1999). These are in the same range as plasma adrenaline concentrations which can be found in the human during birth (Lagercrantz & Bistoletti, 1977; Faxelius *et al*., 1983; Faxelius *et al*., 1984; Greenough *et al*., 1987). Moreover, the adrenaline doses used to evoke increments of exhaled NO (0.1–10 $\mu g\ kg^{-1}\ min^{-1}$) are similar to those used in the human neonate to stabilise systemic the circulation (Levin, 1998).

4.4. HFOV AND PULMONARY NO PRODUCTION (PAPER V)

The main findings of this paper are that

- HFOV significantly increases NO excretion and intratracheal NO concentrations in comparison with intermittent mandatory ventilation in healthy anaesthetised rabbits.
- the stimulation of pulmonary NO production by HFOV is not attributable to disparate $PaCO_2$, mean airway pressure, dead space ventilation or dissimilar increases in functional residual capacity (ΔFRC).
- HFOV also increases endogenous NO production in blood-free buffer-perfused lungs under conditions of constant flow.

The physiological parameters found during measurements of NO and CO_2 excretion are depicted in Tab.3. The mechanism by which the increase of NO excretion during HFOV is effected can not be fully explained on the basis of the present study. However, our experiments rule out a number of potential explanations which are discussed in detail in **PAPER V**. A reasonable explanation of the increase of NO excretion during HFOV would be the existence of stretch-responsive mechanisms which regulate NO production in the lung and its airways and NO release into exhaled gas. Stretch-induced NO formation has, for example, been found in endothelial cells (Hutcheson & Griffith, 1991). Such a mechanism in the lung, the cellular basis of which remains to be shown, could explain all observations made in relation to stretch responses of NO production in the lungs and airways. This hypothesis will be further elaborated below (chapter 5.3). Clearly, further studies on pulmonary NO

production during HFOV in diseased lungs as well as on the optimum mechanical conditions to release NO during artificial ventilation are warranted.

Table 3 Physiological parameters during measurement of NO and CO_2 excretion in ventilated rabbits (n = 23)

	IMV	**HFOV**	**statistical comparison**
Respiratory rate ["breaths" min^{-1}]	40	540	
NO excretion [nl min^{-1}]	9.6 ± 0.8	22.6 ± 2.7	$p \leq 0.001$
NO concentration at outlet [ppb]	1.27 ± 0.11	0.98 ± 0.11	$p < 0.05$
Total gas flow at respirator outlet [L min^{-1}] (including bypass flow)	7.35 ± 0.36	22.88 ± 0.46	$p < 0.001$
Peak airway pressure [mbar]	12.7 ± 0.5	14.5 ± 0.7	$p \leq 0.05$
Mean airway pressure [mbar]	7.3 ± 0.2	4.2 ± 0.3	$p \leq 0.001$
"Tidal" volume [ml]	20.1 ± 0.6	5.6 ± 0.1	$p \leq 0.001$
$PaCO_2$ [kPa]	4.8 ± 0.1	5.5 ± 0.1	$p \leq 0.001$
PaO_2 [kPa]	18.5 ± 0.3	17.0 ± 0.5	n.s.
Mean arterial blood pressure [cm H_2O]	97 ± 5	109 ± 5	$p \leq 0.01$
Heart rate [bpm]	287 ± 7	283 ± 7	n.s.
CO_2 concentration at outlet [%]	0.28 ± 0.05	0.1 ± 0.01	$p < 0.001$
CO_2 excretion [ml min^{-1}]	18.82 ± 1.58	22.35 ±1.29	$p < 0.05$
- " - body-weight related [ml min^{-1} kg^{-1}]	7.6 ± 0.6	9.2 ± 0.7	$p \leq 0.05$
Alveolar ventilation [ml min^{-1}]	395 ± 36	418 ± 26	n.s.

All values given in mean ± SEM
IMV - intermittent mandatory ventilation, HFOV - high frequency oscillatory ventilation

5. GENERAL DISCUSSION - REGULATION OF PULMONARY NO SYNTHESIS

5.1. PULMONARY INFLAMMATION

The usefulness of exhaled NO measurement to monitor ongoing asthmatic inflammation has already been pointed out (chapter 4.1.). However, the regulation of NO formation in asthmatic inflammation needs to be further elaborated. NOS activity is increased in lung samples of patients with inflammatory lung disease (Belvisi *et al*., 1995) and bronchial epithelial cells show increased immunostaining for NOS in biopsy specimens taken from asthmatic patients in comparison with non-asthmatic controls (Hamid *et al*., 1993). Just as in other tissues (cf. chapter 1) pulmonary iNOS is induced by cytokine stimulation, e.g. in bronchial epithelial cells (Asano *et al*., 1994; Robbins *et al*., 1994a; Robbins *et al*., 1994b; Warner *et al*., 1995). These observations have been the basis for a recent hypothesis regarding the pathogenesis of asthmatic inflammation (Barnes & Liew, 1995). According to this hypothesis proinflammatory cytokines (TNF-α, IL-1β, IFN-γ) might, for example, be released from macrophages which may be activated by allergen via low affinity IgE receptors (FcεRII) (Barnes, 1998). The resulting induction of iNOS activity in bronchial epithelial cells results in the prolonged production of large amounts of NO (Guo *et al*., 1997). NO suppresses Th1 cells, reducing their total excretion of IFN-γ, which favours Th2 proliferation (Taylor-Robinson *et al*., 1994). Cytokine release from Th2 cells results in the production of IL-10, which further suppresses Th1-activity, IL-4, which triggers local IgE production and IL-5, which mediates eosinophilic inflammation. Once commenced, eosinophilic asthmatic inflammation will trigger a number of effector systems, namely the 5-lipoxygenase pathway, the release of mast cell proteases and the activation of neutral endopeptidase (NEP) (Drazen *et al*., 1995). These effectors mediate the known clinical changes in bronchial asthma, e.g. bronchoconstriction, mucus secretion and plasma exsudation/mucosal oedema as well as a more chronic inflammatory process characterized by coughing and increased bronchial hyper-responsiveness as well as structural changes (Barnes, 1998). Given the facts that NO can interfere with these effector mechanisms (chapter 1.1.), NO may have a dual function as both initiator and effector in eosinophilic inflammation, which is the hallmark of bronchial asthma. The hypothesis of the role of NO in the initiation of eosinophilic asthmatic inflammation predicts that inhibition of iNOS should alleviate eosinophilic inflammation (Barnes & Liew, 1995). Glucocorticosteroids inhibit iNOS activity *in vitro* in various cell types including bronchial epithelial cells (Di Rosa *et al*., 1990; Radomski *et al*., 1990; Robbins *et al*., 1994a; Robbins *et al*., 1994b). The inhibitory effect of glucocorticosteroids on iNOS is probably mediated by an interaction of cytoplasmic glucocorticoid receptors with the DNA-binding protein NFκB (Adcock *et al*., 1996). In support of the hypothesis outlined above, repeated measurements of exhaled NO in adult asthmatic patients during treatment with inhaled

glucocorticosteroids reveals a gradual decrease of exhaled NO levels which parallels the clinical improvement (Kharitonov *et al*., 1996b).

Apart from asthma there is a wide spectrum of other inflammatory conditions of the conducting airways and lung, e.g. rhinitis, acute and chronic bronchitis, bronchiectasis, emphysema, cystic fibrosis, bronchiolitis, ARDS and fibrosing alveolitis as well as hyaline membrane disease, meconium aspiration syndrome and bronchopulmonary dysplasia in neonates. These disease entities differ markedly in their clinical expression, progressive nature, surgical pathology and response to anti-inflammatory treatment (Pierce & Bancalari, 1995; Viscardi *et al*., 1997; Jeffery, 1998), so that variable effects on exhaled NO, some of which are summarised in Tab.4, are feasible. The comparison of the changes in exhaled NO caused by different inflammatory conditions of the airways and lungs may

Table 4 Some reported effects of pulmonary disease conditions on exhaled NO

DISEASE	REFERENCE
Increased exhaled NO	
atopic bronchial asthma	(chapter 1.2.; **PAPERS I & II**)
acute URTI	(Alving *et al*., 1993b; Kharitonov *et al*., 1995e; de Gouw *et al*., 1998a)
untreated bronchiectasis	(Kharitonov *et al*., 1995d)
COPD	(Kanazawa *et al*., 1998; Maziak *et al*., 1998)
acute graft rejection after lung transplantation	(Silkoff *et al*., 1998a)
Unchanged or decreased exhaled NO	
immotile cilia syndromes	(Lundberg *et al*., 1994c; Karadag *et al*., 1999)
cystic fibrosis	(Balfour-Lynn *et al*., 1996; Dötsch *et al*., 1996; Lundberg *et al*., 1996a; Grasemann *et al*., 1997b; Ho *et al*., 1998a)
bronchiectasis	(Ho *et al*., 1998a)
ARDS	(Brett & Evans, 1998)
COPD	(Rutgers *et al*., 1998)

ARDS – acute respiratory distress syndrome, COPD – chronic obstructive pulmonary disease, URTI – upper respiratory tract infection

be useful to draw conclusions as to the specific nature of the inflammatory process. For example in ARDS, when the degree of acute inflammation is intense (Pittet *et al*., 1997), secondary reactions of NO, e.g. with reactive oxygen species (Chabot *et al*., 1998), may lead to decreased levels in exhaled gas even though iNOS is induced in alveolar macrophages obtained from BAL fluid (Kobayashi *et al*., 1998). In cystic fibrosis an innate deficiency of iNOS enzyme expression in bronchial epithelial cells *in vivo* may explain lacking stimulatory effects of proinflammatory cytokines on pulmonary NO synthesis *in vitro* (Meng *et al*., 1998) and hence decreased NO concentrations in exhaled gas. Alternatively, as nitrite levels in breath condensate of stable cystic fibrosis patients are elevated (Ho *et al*., 1998b), altered NO metabolism or diffusion may explain that exhaled NO levels are not increased in cystic fibrosis patients. Future studies will elucidate the question whether NO is friend or foe in the inflammatory pulmonary diseases named above. In bronchial asthma a dual situation is conceivable in which NO promotes eosinophilic inflammation (references above) but counteracts bronchial hyperresponsiveness (Ricciardolo *et al*., 1996; Mehta *et al*., 1997a; Nogami *et al*., 1998; Taylor *et al*., 1998b; Kanazawa *et al*., 1999). Studies on the benefit of therapeutic interventions into NO metabolism will most likely resolve this. Meanwhile, given further technical improvement and standardisation, the clinical value of exhaled NO analysis for monitoring anti-asthmatic therapy may be firmly established.

5.2. ADRENOCEPTOR STIMULATION

Beta-adrenoceptor agonists, mainly adrenaline, regulate a number of functions in the respiratory tract - β-adrenoceptors are expressed on airway smooth muscle cells, mast cells, subepithelial glands, bronchial epithelium and cholinergic ganglia (Barnes, 1988). Bronchial epithelial cells have been shown to increase their basal NO excretion in response to β-adrenoceptor stimulation of ciliary motility (Tamaoki *et al*., 1995a). Considering the known vasodilative effect of NO on the pulmonary circulation (Ignarro *et al*., 1988), our results suggest the possibility that β-adrenoceptor action on the pulmonary circulation could be mediated by NO. Similar observations have been made in other tissues and parts of the circulation: β_2-mediated vasodilation is dependent on NO synthesis in human forearm musculature (Dawes *et al*., 1997). In the bronchial circulation of sheep, the vasodilative effects of isoetharine, a β-adrenergic agonist with β_2-selectivity, is predominantly mediated by NO (Charan *et al*., 1997), while an involvement of the β_1-adrenoceptor subtype has been claimed in rats (Corboz *et al*., 1996). Similarly, β_2-mediated vasodilation is partly dependent on NO synthesis in canine coronary resistance vessels (Ming *et al*., 1997). In piglet pial arterioles both β_1- and β_2-mediated vasodilation is dependent on an intact NO synthesis (Rebich *et al*., 1995). In contrast to our findings (**PAPER IV**) direct activation of adenylyl cyclase by application of forskolin also elicited increases of cGMP in

periarachnoid CSF which could be prevented by NOS inhibition (Rebich *et al*., 1995). Hence the novelty of our findings lies in the fact that NO production seems to be stimulated mainly via β_1-receptors and, secondly, that the transduction of this signal does not seems to involve formation of cAMP.

Beta-adrenergic mechanisms are essential during postnatal pulmonary adaptation to extrauterine life. Apart from well-known effects on the pulmonary circulation, β-adrenoceptor activation also stimulates surfactant production and release (Lawson *et al*., 1978; Olver, 1981) and enhances the resorption of lung water from alveolar epithelial cells via stimulation of Na^+-K^+-ATPase activity, amiloride–sensitive Na^+-channels and non-selective cation channels (Berthiaume *et al*., 1999). It is generally accepted that it is the β_2 subclass of adrenoceptors that are mediating this effect. However, a review of the relevant literature (Berthiaume *et al*., 1999) shows that β_1- and β_2- selective agents have not been compared in a controlled fashion. Catecholamine concentrations in plasma increase about 10-fold from the first stage of labour until birth and are then in fact at least as high as at any time point later during life (Lagercrantz & Slotkin, 1986; Lagercrantz, 1998). The clinical relevance of this surge is illustrated by a dose-response relationship between catecholamine concentrations in umbilical arterial blood at birth and dynamic lung compliance at the age of 2 hours (Faxelius *et al*., 1983). Moreover, infants of mothers treated with β-adrenergic agonists (terbutaline) have less respiratory compromise in comparison with the infants of non-treated mothers (Bergman & Hedner, 1978). Significantly lower levels of plasma catecholamines have been found after elective caesarean section (Lagercrantz, 1998) so that one can speculate about a causal link to an increased incidence of respiratory compromise in these newborns (Hansen & Corbet, 1998). Similarly, the observations that male neonatal rabbits have a significantly delayed maturation of both adrenal medullary and pulmonary β-adrenoceptors (Padbury *et al*., 1981) and that male human preterms tend to have lower plasma catecholamines than their female counterparts following asphyxia (Greenough *et al*., 1987) fit well with the known clinical fact that the incidence of neonatal respiratory disease is higher in male compared with female infants (Hansen & Corbet, 1998). Seen in the context of this evidence, our study suggests that perinatally high serum catecholamine concentrations might stimulate pulmonary NO synthesis, which is known to be essential for the circulatory transition to extrauterine life (chapter 1.1.). Studies on a possible correlation of plasma catecholamines and pulmonary NO production in the neonatal period clearly are warranted.

5.3. MECHANICAL STRETCH

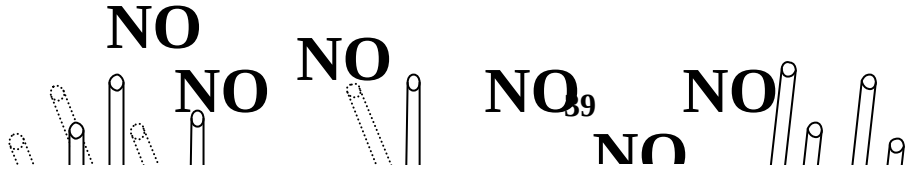
The application of positive end-expiratory pressure (PEEP) elicits an immediate increase of exhaled NO in ventilated rabbits which reaches a peak within seconds and then stabilises at a slightly lower

plateau level (Persson *et al*., 1995; Strömberg *et al*., 1997). This phenomenon is also seen in guinea pigs and can be inhibited by infusion of gadolinium chloride ($GdCl_3$) in both rabbits and guinea pigs (Bannenberg & Gustafsson, 1997; Adding *et al*., 1998). Gadolinium is regarded as a fairly selective inhibitor of stretch-activated calcium channels (Swerup *et al*., 1991; Bialecki *et al*., 1992; Naruse & Sokabe, 1993; Laine *et al*., 1994). Therefore, a stretch-sensitive component of exhaled NO has been postulated. Recently, also increases of tidal volume and prolongation of the inspiratory time have been shown to increase levels of exhaled NO in rabbits (Forsberg *et al*., 1999). Just like the application of PEEP, these manoeuvres are bound to alter the intensity and duration of mechanical stretch exerted on the pulmonary tissues. Stimulatory effects of PEEP on exhaled NO can be reproduced by other ventilator modes, provided that the alteration of peak transpulmonary pressure is comparable (Strömberg *et al*., 1997), again supporting the concept of stretch-sensitive NO release from the lower airways. The fact that even negative extrathoracic pressure can evoke an increase of exhaled NO makes it unlikely that changes in pulmonary arterial pressure can account for this increase (Strömberg *et al*., 1997). An even stronger stimulation of pulmonary NO production in the rabbit can be achieved with HFOV, the mechanical properties of which are characterised by very small tidal volumes being applied at very high rates (**PAPER V**). Mechanical stretch of the pulmonary tissues might also explain the known increases of NO excretion during exercise and during hyperventilation at rest (cf. Tab.2).

The increases of exhaled NO during the application of PEEP have been suggested to be influenced by vagally mediated mechanisms (Persson *et al*., 1995). They have however also been seen in isolated perfused lungs (Carlin *et al*., 1997a), when an intact neurotransmission involving afferent and efferent nerves is questionable. Functional neurons may thus not be necessary to evoke increases of NO production in response to mechanical stimuli. Indeed there is circumstantial evidence to suggest the existence of cellular mechanotransduction regulating NO synthesis and release. In endothelial cells, mechanical stimulation upregulates eNOS expression (Awolesi *et al*., 1995) and a maximum of NO release is reached at low amplitudes and high rates of mechanical stimulation (Hutcheson & Griffith, 1991). The link of increased NO production to mechanical stimulation may be mediated by elevations of cytosolic Ca^{2+} concentrations, which are known to be increased by cyclic strain in endothelial cells (Rosales *et al*., 1997). A similar stretch-induced increase of intracellular Ca^{2+} concentrations is seen in bronchial epithelial cells (Sanderson *et al*., 1990; Wirtz & Dobbs, 1990), in which at least two stretch activated channels have been identified (Wirtz & Dobbs, 1990; Kim *et al*., 1993). As mechanical stimulation of a bronchial epithelial cell not only increase intracellular calcium concentration in this cell but also in the surrounding epithelial cells via intercellular communications (Sanderson *et al*., 1990), a relatively wide-spread stimulating effect on NOS is conceivable. Exhaled NO levels are known to be partially dependent on the extracellular presence of free calcium ions (Persson *et al*., 1994b). A hypothesis on the link between mechanical stretch and increased pulmonary NO production resulting in

increases of exhaled NO is depicted in Fig.1. This hypothesis is based on the reasonable assumption (chapter 1.2.) that exhaled NO is likely to be mainly derived from bronchial epithelial cells. Due to the intracellular network of microtubules and microfilaments, which are attached to the cell membrane, an external mechanical stimulus is redistributed over the entire cytoskeletal scaffolding. Ion channels can for example be regulated by cytoskeletal interconnections (Yang & Sachs, 1993), thus converting a mechanical signal into a chemical one. The subcellular structure of focal adhesion complexes (FAC) may represent a major site for this conversion (Davies, 1995; Ingber, 1997). According to the evolving concept of tensegrity, they may thus be the molecular basis of mechanotransduction without the need for a specific cellular stretch receptor (Ingber, 1997). Phosphatidylinositol kinases, i.e. the enzymes providing phosphatidyl bisphosphate for further intracellular signalling, are immobilised on the cytoskeleton within the FAC (Ingber, 1997). This probably explains the observation that the enzyme activity of phospholipase C (PLC) is stretch-responsive. PLC creates inositol trisphosphate, which ultimately leads to release of Ca^{2+} ions from the endoplasmatic reticulum. This may in turn stimulate NOS activity in the cytoplasm. In addition to FAC another cellular structure, namely the cilia, are structurally attached to the cytoskeleton (Philippou *et al*., 1993), so that ciliary beating will exert a mechanical stimulus on the ciliary base and the cytoskeleton during ciliary beating. NOS has been detected within the basal ciliary plate (Xue *et al*., 1996). It is therefore conceivable that the mechanical stimulus of ciliary beating may stimulate NOS activity in the basal plate. This assumption predicts that a functional defect preventing proper ciliary movements should fail to stimulate NO production. Indeed, exhaled NO is significantly decreased in patients with dysfunctional cilia (Lundberg *et al*., 1994c; Karadag *et al*., 1999). Conversely, pharmacological stimulation of ciliary beating by a β-adrenoceptor agonist leads to an immediate increase of NO release from cultured bronchial epithelial cells (Tamaoki *et al*., 1995b). Summing up, there is evidence to suggest a stimulating influence of mechanical stretch and ciliary activity on pulmonary NO production. Regardless of uncertainties about the cellular basis of this phenomenon, future studies will have to assess its relative importance in the human and the possibility that modifications of current regimens of respirator treatment may exploit this mechanism for the benefit of the patient.

Figure 1
Hypothesis of NO regulation in response to mechanical stretch or ciliary stimulation in bronchial epithelial cells



Dashed cell outline indicates tentative change of cell shape caused by stretching of the airways

closed circles - NOS enzyme

open circles - 1 phosphatidyl inositol kinase

- 2 phospholipase C (PLC)

FAC - Focal adhesion complex, ECM - extracellular matrix, ER - endoplasmatic reticulum

PIP - phosphatidylinositol 4 phosphate, PIP_2 - phosphatidylinositol 4,5 bisphosphate

IP_3 - inositol 1,4,5-trisphosphate

6. SUMMARY & CONCLUSIONS

1. Nitric oxide (NO) in gas exhaled from the lower respiratory tract can be reliably measured in newborn infants and children, achieving good repeatability and distinguishing contributions derived from the nose.

2. Asthmatic children display significantly greater amounts of NO in mixed orally exhaled gas than healthy control subjects in spite of pulmonary function parameters in the normal range. This suggests that exhaled NO may be an early signal of asthmatic airway inflammation.

3. Recent clinical symptoms of airflow obstruction are linked to increased amounts of NO in mixed orally exhaled gas in spite of unchanged pulmonary function parameters. This suggests that exhaled NO measurement can be useful to monitor recent clinical symptoms and the effectiveness of anti-inflammatory treatment in childhood asthma.

4. The concentration range of autoinhaled NO in newborn infants advocates biological effects of NO in the lower respiratory tract of the newborn. Transient shortage of autoinhaled NO due to temporary decreases in autoinhaled NO concentrations might be of functional significance.

5. Adrenoceptor stimulation can increase pulmonary NO production in the rabbit. This effect on pulmonary NO production can be mediated by β_1-adrenergic mechanisms and may be important to limit pulmonary artery pressure during stress and the circulatory transition at birth.

6. High frequency oscillatory ventilation stimulates pulmonary NO production in rabbits. Increased stretch activation of the respiratory system during HFOV is suggested as a possible underlying mechanism. The physiological importance of the increased NO is unknown. It might serve to improve ventilation perfusion matching and regulate bronchial function.

A. Artlich

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9. APPENDIX (PAPERS I – V)