LAURI LEHTIMÄKI

Measurement of Exhaled Nitric Oxide

Differentiation between Alveolar and Bronchial Inflammation by Using Multiple Exhalation Flow Rates

■

ACADEMIC DISSERTATION

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ABSTRACT

Chronic inflammation is a key feature in many lung diseases, for example asthma. Currently, the diagnosis and follow-up of inflammatory lung diseases are based largely on radiography and measurements of lung function. However, these methods do not measure the inflammatory process but secondary changes caused by it. Direct measures of inflammation would therefore provide more precise information on the underlying pathogenetic process and the activity of the disease.

Nitric oxide (NO) is an endogenous signalling molecule in the lungs, produced in small amounts in the physiological state. In inflammatory lung diseases, pulmonary NO production increases and inflammation can be detected based on an increased concentration of NO in exhaled breath. Exhaled NO concentration is usually measured at repeated exhalations at a single low flow rate. This method can be used to detect pulmonary inflammation, but it cannot discriminate between alveolar and bronchial contributions to exhaled NO, and therefore cannot differentiate between alveolar and bronchial components in the inflammatory process. Mathematical models of pulmonary NO dynamics suggest that the alveolar and bronchial contributions to exhaled NO concentration can be calculated on the basis of exhaled NO measurements at multiple different exhalation flow rates. The aim of the present study was to establish whether assessment of alveolar and bronchial NO output could be used to differentiate between alveolar and bronchial inflammation.

Alveolar NO concentration and bronchial NO flux were calculated in steroid-naïve patients with asthma, patients with alveolitis, and healthy subjects. Asthmatics had increased bronchial NO flux but on average normal alveolar NO concentration, whereas patients with alveolitis showed increased alveolar NO concentration but normal bronchial NO flux. In asthma and alveolitis, bronchial NO flux and alveolar NO concentration, respectively, correlated with markers of disease severity. Anti-inflammatory treatment reduced the bronchial NO flux in asthma and the alveolar NO concentration in alveolitis simultaneously with improvement of the disease state. Alveolar NO concentration was also increased in asthmatics with nocturnal symptoms, which is consistent with the peripheral inflammation detected in nocturnal asthma. Alveolar and bronchial NO output were also increased in subjects presenting with asthmatic symptoms but not fulfilling the lung function criteria for asthma.

It is concluded that assessment of alveolar and bronchial NO output by measuring exhaled NO concentration at multiple exhalation flow rates can be used to differentiate between the alveolar and bronchial components of pulmonary inflammation. Exhaled NO measurement at multiple flow rates provides more detailed information on the inflammatory process than the currently recommended single flow rate method.

ABBREVIATIONS

ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
C_{Alv}	Steady state nitric oxide concentration in alveolar air [ppb]
CFA	Cryptogenic fibrosing alveolitis
cGMP	Cyclic guanosine monophosphate
cNOS	Constitutive nitric oxide synthase
COPD	Chronic obstructive pulmonary disease
C_W	Nitric oxide concentration in bronchial wall tissue [ppb]
$D_{NO,Alv}$	Alveolar diffusing capacity of nitric oxide [nl/s/ppb]
$D_{NO,Br}$	Bronchial wall diffusing capacity of nitric oxide [nl/s/ppb]
DL_{CO}	Pulmonary diffusing capacity of carbon monoxide [ml/s/mmHg]
DRS	Dose-response slope
EAA	Extrinsic allergic alveolitis
ECP	Eosinophil cationic protein
eNOS	Endothelial nitric oxide synthase
EPX	Eosinophil protein X
FASSc	Fibrosing alveolitis associated with systemic sclerosis
$FEF_{50\%}$	Forced expiratory flow when 50 % of VC has been exhaled [l/s]
$FEF_{75\%}$	Forced expiratory flow when 75 % of VC has been exhaled [l/s]
FEV_1	Forced expiratory volume in 1 second [l]
FVC	Forced vital capacity [l]
HRCT	High-resolution computed tomography
hз	Energy of electromagnetic radiation, where h is Planck's coefficient and з is the frequency of the radiation
IgE	Immunoglobulin E
IL-1	Interleukin 1
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
$J_{NO,Br}$	Maximal diffusion rate of NO from bronchial wall to luminal air, bronchial NO flux [nl/s]
$J_{NO,Alv}$	Diffusion rate of NO from alveolar tissue to alveolar air during steady state [nl/s]
LTE_4	Leukotriene E_4
MPO	Myeloperoxidase
NF-ρB	Nuclear factor ρB
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2	Nitrogen dioxide
O_2	Molecular oxygen
O_3	Ozone
p	Probability value
$PD_{20}FEV_1$	Provocative dose causing a 20 % decrease in FEV_1
PEF	Peak expiratory flow [l/min]
r	Pearson's correlation coefficient
SEM	Standard error of mean
TNF-ζ	Tumour necrosis factor ζ
UIP	Usual interstitial pneumonia
VC	Vital capacity [l]
$\dot{V}_{NO}$	Total nitric oxide output from lower respiratory tract [nl/s]
$\dot{V}$	Exhalation flow rate [l/s]

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by their Roman numerals (I – V). In addition, some unpublished data are presented.

I. Lehtimäki L, Turjanmaa V, Kankaanranta H, Saarelainen S, Hahtola P and Moilanen E (2000). Increased bronchial nitric oxide production in patients with asthma measured with a novel method of different exhalation flow rates. *Annals of Medicine*, 32: 417-423.

II. Lehtimäki L, Kankaanranta H, Saarelainen S, Hahtola P, Järvenpää R, Koivula T, Turjanmaa V and Moilanen E (2001). Extended exhaled NO measurement differentiates between alveolar and bronchial inflammation. *American Journal of Respiratory and Critical Care Medicine*, 163: 1557-1561.

III. Lehtimäki L, Kankaanranta H, Saarelainen S, Turjanmaa V and Moilanen E (2001). Inhaled fluticasone decreases bronchial but not alveolar nitric oxide output in asthma. *European Respiratory Journal*, 18: 635-639.

IV. Lehtimäki L, Kankaanranta H, Saarelainen S, Turjanmaa V and Moilanen E (2002). Increased alveolar nitric oxide concentration in asthmatic patients with nocturnal symptoms. *European Respiratory Journal*, 20: 841-845.

V. Lehtimäki L, Kankaanranta H, Saarelainen S, Turjanmaa V and Moilanen E. Peripheral inflammation in patients with asthmatic symptoms but normal lung function. Submitted for publication.

1. INTRODUCTION

Inflammation is an important host response elicited by tissue injury or invading pathogens. Although inflammation is a vital defence response, e.g. during infection, chronic inflammation also has many detrimental effects. Inflammation is a key feature in the pathogenesis of many lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), extrinsic allergic alveolitis (EAA) and cryptogenic fibrosing alveolitis (CFA). The prevalence of asthma and COPD is increasing, and these diseases constitute a major burden to patients and health care organisations in many countries. Inflammatory lung diseases are nowadays mainly diagnosed using radiography and indicating impaired pulmonary function, a secondary change caused by chronic inflammation. There is, however, a clear need for widely accessible direct measures of pulmonary inflammation to allow more precise diagnosis and follow-up of inflammatory lung diseases. Such measures would also facilitate the assessment of the efficacy of anti-inflammatory treatment. During recent years, considerable interest has focused on developing novel non-invasive methods to measure pulmonary inflammation through analysis of inflammatory markers in exhaled breath.

Nitric oxide (NO) is an endogenous signalling molecule produced in the human body. In the physiological state, low amounts of NO are produced for regulatory purposes. In inflammation, on the other hand, NO production increases and induces cytotoxic effects against both invading organisms and host cells. A small fraction of the NO synthesised in the lungs diffuses into the pulmonary air and endogenous NO can be detected in exhaled air. In inflammatory lung diseases, pulmonary NO production increases considerably and the inflammatory process can be detected on the basis of the increased NO concentration in exhaled air. The interest in exhaled NO measurement as a non-invasive measure of inflammation has been intense, and exhaled NO measurement is now considered as a promising novel tool in the diagnosis and follow-up of various inflammatory lung diseases. The aim of the present study was to further develop exhaled NO measurement by using a mathematical model of pulmonary NO dynamics, which provides a theoretical means to assess alveolar and bronchial components in pulmonary inflammation separately.

2. REVIEW OF THE LITERATURE

2.1 Nitric Oxide

Nitric oxide (NO) is a small diatomic molecule formed by nitrogen and oxygen atoms covalently bound to each other. It is a colourless gas at room temperature and pressure. NO has one unpaired electron and is thus a highly reactive molecule.

NO was first discovered as an endogenous signalling molecule in 1987, when the endothelium-derived relaxing factor, an important vasodilatory signalling molecule, was revealed to be NO (Ignarro et al. 1987, Palmer et al. 1987). Since then, this small gaseous molecule has been found to play a crucial role in human physiology and pathophysiology. NO is an important regulator of blood pressure and platelet aggregation, it acts as a neurotransmitter in both central and peripheral nervous systems and regulates immune responses (Moncada et al. 1991, Christopherson and Bredt 1997, Marin and Rodriguez-Martinez 1997, Moilanen et al. 1999, Bogdan 2001). In 1998, the Nobel Prize for Physiology or Medicine was awarded to three pharmacologists, Robert R. Furchgott, Louis J. Ignarro and Ferid Murad, for their fundamental work in exploring the role of NO in cardiovascular physiology.

2.1.1 Synthesis of NO

NO is synthesised from amino acid L-arginine by a family of nitric oxide synthase (NOS) enzymes. The reaction catalysed by NO synthases requires oxygen and NADPH to convert L-arginine into NO and the amino acid citrulline. NO synthases are dimeric haeme containing enzymes composed of oxygenase and reductase domains which possess binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin (CaM) and tetrahydrobiopterin (BH_4) (Janssens et al. 1992, Lowenstein et al. 1992, Knowles and Moncada 1994, Alderton et al. 2001) (Figure 1).

Three NOS isoforms encoded by distinct genes are known to date, namely neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Although originally found in neuronal and endothelial cells, nNOS and eNOS are expressed in a variety of cell types throughout the human body (Föstermann et al. 1998, Alderton et al. 2001).

nNOS and eNOS are so-called constitutive NO synthases (cNOS), i.e. they are expressed in several cell types in response to physiological stimuli, and they are thought to produce the fairly low amounts of NO needed in different physiological processes (Kleinert et al. 2000). After expression, the enzymatic activity of the constitutive NO synthases is strictly regulated by the intra-cellular Ca^{2+} concentration (Bredt and Snyder 1990, Busse and Mulsch 1990, Alderton et al. 2001). eNOS- and nNOS-dependent NO production in endothelial and neural cells is activated by signalling molecules such as acetylcholine, bradykinin and glutamate, which increase the intracellular Ca^{2+} concentration through receptor-associated

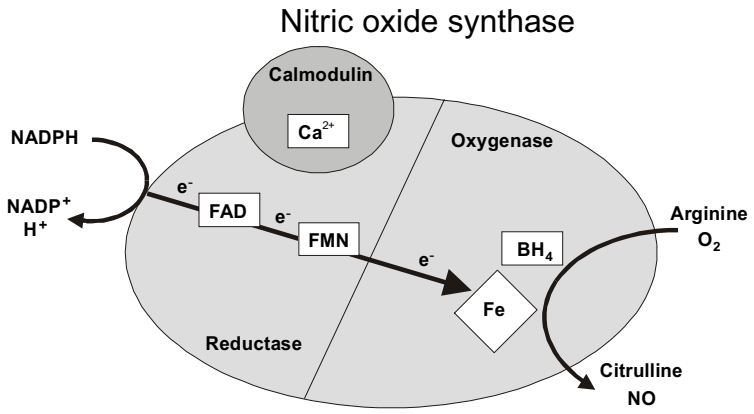

Figure 1. Nitric oxide synthase, its cofactors and the overall reaction catalysed. Electrons are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN to the oxygenase domain. There they interact with the haem iron and BH_4 at the active site to catalyse the reaction of oxygen with L-arginine, generating citrulline and NO as products. Electron flow through the reductase domain requires the presence of bound Ca^{2+} / calmodulin (Alderton et al. 2001).

mechanisms (Johns et al. 1987, Garthwaite et al. 1988, Christopherson and Bredt 1997, Marin and Rodriguez-Martinez 1997).

By contrast, iNOS is not typically present in the physiological state. Its expression is induced in many cell types by bacterial products (e.g. endotoxin) and inflammatory cytokines such as IL-1, IFN-ν or TNF-ζ (Kleinert et al. 2000, Alderton et al. 2001). Once expressed, iNOS produces NO for prolonged periods. iNOS produces NO in much higher quantities as compared with cNOS (Kleinert et al. 2000).

iNOS differs from the constitutive enzyme forms also in that its expression is suppressed by anti-inflammatory treatment with glucocorticoids, whereas cNOSs are not steroid-sensitive (Radomski et al. 1990). Glucocorticoids have been shown to reduce iNOS expression by inhibiting NF-ρB, an important transcription factor for iNOS (Kleinert et al. 1996), and by undermining the stability of iNOS mRNA (Korhonen et al. 2002).

A variety of different NOS inhibitors have been developed and used as research tools to extend our knowledge of the physiological role of NO. Most of these inhibitors bind at the arginine-binding site of the enzyme and act competitively (Alderton et al. 2001). Some of the inhibitors, for example L-NMMA (N^G-monomethyl-L-arginine), inhibit all NOS isoforms with similar potency, whereas others are isoform-specific and provide possibilities to study the role of different isoforms. iNOS-specific inhibitors, e.g. L-NIL (L-*N6*-(1-iminoethyl)lysine), are widely used to study the role of iNOS-dependent high NO production in inflammatory and other conditions.

2.1.2 Signal transduction by NO

Unlike the classical intercellular signalling molecules, NO has no specific receptor mediating its action. Being a small gaseous molecule it diffuses easily through cell membranes and alters cell function by affecting cellular proteins. Signal transduction by NO can be divided into direct and indirect mechanisms. In direct effects, NO itself reacts with the key molecules to mediate the biological function. In indirect effects, on the other hand, NO first reacts with other molecules like oxygen or superoxide to form compounds such as peroxynitrite, which in turn react with the target molecules (Grisham et al. 1999). At low physiological concentrations, the direct mechanisms of NO signalling predominate. In states of inflammation, where iNOS is expressed and high amounts of NO are produced, indirect effects are thought to take over (Miranda et al. 2000) (Figure 2).

NO reacts directly with many enzymes, and the most important pathway in its physiology is the activation of guanylate cyclase (Arnold et al. 1977, Grisham et al. 1999). Activation of guanylate cyclase leads to increased cGMP production in target cells, mediating e.g. relaxation of smooth muscle in blood vessels and airways and inhibition of platelet aggregation (Furlong et al. 1987, Ignarro et al. 1987, Palmer et al. 1987, Pohl and Busse 1989). NO also reacts avidly with oxyhaemoglobin, forming nitrate and methaemoglobin (Doyle and Hoekstra 1981, Miranda et al. 2000). This is an important inactivation route of NO in the body.

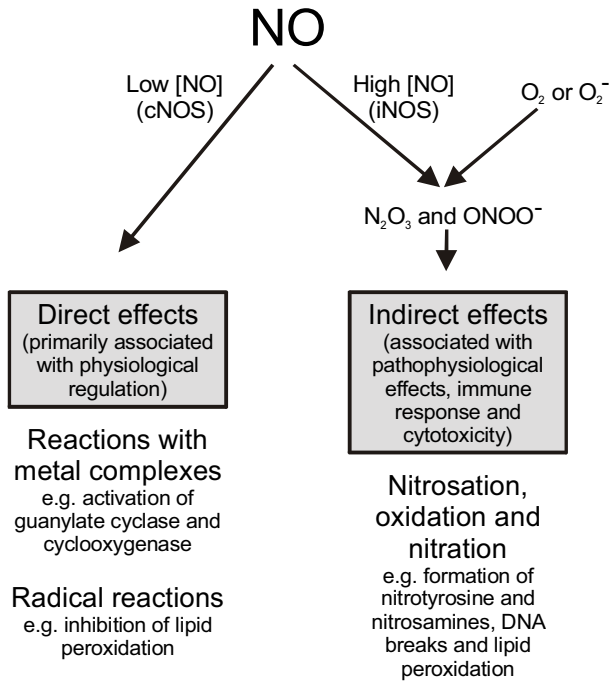

Figure 2. Direct and indirect effects of NO. Modified from Grisham et al. (1999) and Miranda et al. (2000). O_2^-, superoxide; N_2O_3, dinitrogen trioxide; $ONOO^-$, peroxynitrite.

NO reacts with molecular oxygen (O_2) and superoxide anion (O_2^-) to form reactive nitrogen species such as dinitrogen trioxide (N_2O_3) and peroxynitrite ($ONOO^-$), which mediate the indirect effects of NO. Formation of peroxynitrite is abundant in inflammatory states associated with an increased production of superoxide anion. Dinitrogen trioxide causes nitrosation of amines and thiols, whereas peroxynitrite causes nitration of e.g. tyrosine residues. Peroxynitrite is also a powerful oxidant which initiates lipid peroxidation and cleaves DNA (Pryor and Squadrito 1995, Beckman and Koppenol 1996, Wink et al. 1997). NO in small concentrations regulates mitochondrial respiration by reversibly inhibiting cytochrome oxidase, the terminal complex of the mitochondrial electron transport chain (Brown and Cooper 1994, Cleeter et al. 1994). At higher NO concentrations peroxynitrite is formed, and irreversibly inhibits several complexes of the respiratory chain and therefore hampers mitochondrial respiration (Lizasoain et al. 1996). This may play a significant role in causing tissue damage in disease states such as ischemia-reperfusion injury and endotoxin-induced or haemorrhagic shock (Liaudet et al. 2000). These indirect effects are thought to mediate most of the detrimental and cytotoxic effects of NO in inflammation (Grisham et al. 1999, Miranda et al. 2000).

2.1.3 NO in inflammation

Nitric oxide plays a dual role in the inflammatory process. Constitutive low NO production exerts many protective effects against inflammatory changes, whereas the increased NO and peroxynitrite production following upon iNOS expression is associated with cytotoxicity and potentiation of many detrimental events.

Constitutive NO production by eNOS has a protective role against microcirculatory damage and oedema formation during the early phase of inflammation. After a few hours iNOS is expressed in vascular cells. High amounts of NO produced by iNOS exacerbate oedema and iNOS inhibition has been shown to reduce microvascular leakage in inflammation (Boughton-Smith et al. 1993, Laszlo et al. 1994, Moilanen et al. 1999).

NO also exerts anti-inflammatory effects by reducing leukocyte extravasation. Endogenous NO production or NO-releasing drugs reduce the expression of adhesion molecules in endothelial cells and thereby suppress leukocyte adhesion to the endothelium and their extravasation (Kubes et al. 1991, Kosonen et al. 2000).

NO suppresses lymphocyte proliferation in a GMP-independent manner (Albina et al. 1991, Kosonen et al. 1997, Kosonen et al. 1998a). It might also participate in regulation of the Th_1/Th_2 lymphocyte balance, as it inhibits IL-2 and IFN-ν secretion by Th_1 cells but has no effect on the cytokine production of Th_2 cells (Taylor-Robinson et al. 1994). NO would therefore favour the Th_2 response and might participate in the development of atopic diseases.

Inflammatory cytokines and microbial products induce iNOS expression in macrophages (Lyons et al. 1992, Xie et al. 1992, Bogdan 2001, Thomassen and Kavuru 2001). The high amount of NO produced is

thought to play an important role in cytostatic-cytotoxic effects of macrophages against tumour cells and microbes (MacMicking et al. 1997, Hibbs et al. 1988). Induction of iNOS expression is better known in rodent than in human macrophages, but the latter also have been reported to express iNOS after a variety of stimuli (Moilanen et al. 1997, Thomassen and Kavuru 2001). Human alveolar macrophages express iNOS in patients with cryptogenic fibrosing alveolitis (Saleh et al. 1997) or after induction by e.g. mycobacteria (Nicholson et al. 1996, Nozaki et al. 1997).

The high NO level prevailing in inflammation are thought to exert cytotoxic effects not only against invading microorganisms but also against host cells. Many of these aspects are mediated by peroxynitrite and N_2O_3 formed by reactions between NO and superoxide or O_2. These compounds cause oxidative stress and nitrosylation of many important host proteins, leading e.g. to inhibition of cellular respiration and cell necrosis (Szabo 2000).

NO can also modulate the inflammatory response by altering the activity of other mediator pathways. NO reduces leukotriene synthesis by inhibiting 5-lipoxygenase (Moilanen et al. 1993, Kanner et al. 1992, Maccarrone et al. 1996, Brunn et al. 1997). NO has been reported either to inhibit or activate cyclo-oxygenase (COX), which converts arachidonic acid into prostanoids (Salvemini et al. 1993, Kosonen et al. 1998b, Stadler et al. 1993). The effect of NO on COX activity seems to be concentration-dependent such that low NO amounts activate COX while higher levels inhibit COX activity (Liaudet et al. 2000). NO might thus have important regulatory effects on inflammation in affecting the activity of these lipid mediator pathways.

2.2 NO in Inflammatory Lung Diseases

Inflammation is an essential host response to a variety of insults. It constitutes a host defence against microorganisms and is also necessary to tissue repair after mechanical or chemical injury. Inflammation involves a complex interplay between structural and inflammatory cells and includes a wide variety of mediators needed for inter-cell signalling (Cotran et al. 1999).

Chronic inflammation also has detrimental effects and constitutes a key feature in many pulmonary diseases. Sustained inflammation may cause functional and structural changes such as airway hyperresponsiveness, airway wall thickening, alveolar wall destruction and parenchymal fibrosis (Keane et al. 2000). Early suppression of the inflammatory process is believed to prevent these injuries.

2.2.1 Asthmatic inflammation

Asthma is defined as a *chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells* (National Heart Lung and Blood Institute 1997). The complex inflammatory process underlying asthma can be divided into fairly distinct components, which are responsible for the different

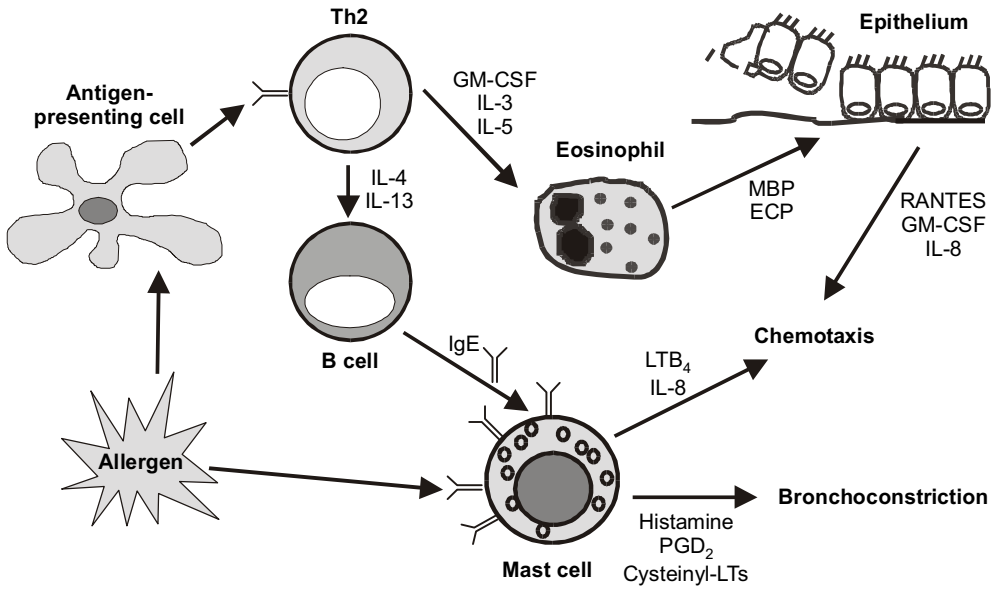

Figure 3. Asthmatic inflammation is a complex interplay of various inflammatory cells and signalling molecules (Boushey et al. 2000). GM-CSF, granulocyte-macrophage colony-stimulating factor; LTB_4, leukotriene B_4; MBP, major basic protein; PGD_2, prostaglandin D_2; RANTES, regulated on activation, normal T cell expressed and secreted.

clinical characteristics of the disease. These are the acute IgE-mediated allergic reaction, chronic inflammation, and airway remodelling (Bousquet et al. 2000).

In sensitised subjects, inhaled allergens bind to specific IgE molecules on the cell surface receptors of different inflammatory cells, particularly mast cells, which become activated and release proinflammatory substances such as histamine and eicosanoids. These substances lead to bronchoconstriction, mucus secretion and vasodilatation, and may provoke an acute asthmatic attack. In the so-called late-phase reaction 6 to 9 hours after the allergen challenge, many inflammatory cell types are recruited into the airways. These include eosinophils, CD4+ T cells, basophils, macrophages and neutrophils (Boushey et al. 2000, Bousquet et al. 2000).

In addition to allergic reactions, chronic airway inflammation is a key feature in asthma. Eosinophils and Th_2 cells play a major role in this condition (Boushey et al. 2000, Bousquet et al. 2000), but also neutrophils are present in great numbers especially in the severe, steroid-resistant form (Wenzel et al. 1997, Jatakanon et al. 1999b). Chronic inflammation causes non-specific airway hyperresponsiveness and structural changes such as an increase in smooth muscle mass and number of mucous glands, epithelial shedding and thickening of the basement membrane (Jeffery 1998, Bousquet et al. 2000). It is thought that early intervention with anti-inflammatory treatment turns off the chronic inflammatory

process and thereby prevents permanent structural changes in asthmatic airways.

Asthmatic inflammation appears to affect the whole lower respiratory tract. Although asthma has classically been thought to affect mainly the large airways, there are data suggesting the presence of inflammation also in the small peripheral airways and alveolar tissue in stable chronic asthma (Kraft et al. 1996). Furthermore, night-time enhancement of alveolar inflammation has been associated with nocturnal asthma (Martin et al. 1991, Kraft et al. 1996, Kraft et al. 1999).

2.2.2 Inflammation in cryptogenic fibrosing alveolitis

Cryptogenic fibrosing alveolitis (CFA), as the name implies, is an inflammatory and fibrosing condition of unknown origin affecting the pulmonary parenchyma. Its pathophysiology involves a combination of tissue damage, inflammation and excess fibrosis. Although the initial damaging agent is not known, several predisposing factors have been suggested. These include a number of organic and inorganic dusts, viral factors and smoking (American Thoracic Society and European Respiratory Society 2000).

The inflammatory cells involved in the pathogenesis of CFA include macrophages, neutrophils, eosinophils and lymphocytes. Fibroblasts and myofibroblasts are responsible for producing the excess collagen characteristic of the disease. Especially macrophages, but also other inflammatory cells, are capable of producing cytokines which can increase collagen synthesis and induce proliferation of fibroblasts. Inflammatory cells also secrete collagenases and thereby regulate collagen turnover. The imbalance between synthesis and degradation of collagen resulting from altered secretion of collagenases and profibrotic substances is considered a central factor underlying the pathogenesis of CFA (Chan et al. 1998, King 1998).

The histopathological features of the disease vary between cellular and fibrotic patterns, where inflammatory and fibrotic changes, respectively, are the major characteristics at microscopic level. The cellular pattern is usually associated with better prognosis and response to drug treatment (American Thoracic Society and European Respiratory Society 2000).

2.2.3 Inflammation in extrinsic allergic alveolitis

Extrinsic allergic alveolitis (EAA) is an inflammatory reaction caused by an immunological response to inhaled organic dusts. The inflammation in this condition affects alveolar, bronchiolar and peribronchiolar tissue. There are many names for EAA depending on the offending antigen and the setting in which the exposure takes place. The most common of these is farmers' lung, caused by exposure to mouldy hay, straw or grain in agriculture. The inhaled allergens are spores of thermophilic actinomycetes, particularly *Micropolyspora faeni* and *Thermoactinomyces vulgaris*. Other examples of EAA are bird fanciers' lung, caused by inhalation of pigeon serum proteins in their bloom, and malt workers'

lung caused by inhalation of spores from mouldy malts. However, the histopathological features of EAA are the same regardless of the causative antigen (Selman 1998).

The characteristic pathological findings in EAA are peribronchiolar granulomatous inflammation and bronchiolocentric alveolitis, characterised by an accumulation of plasma cells, T lymphocytes and macrophages in the inflammatory exudates (Selman 1998). The immunopathogenesis is thought to be driven by immunocomplexes formed by the antigen and specific IgG molecules. Activated T lymphocytes are thought to play a key role in the formation of granulomas. However, neither of these is specific to EAA, as both increased T cell counts in BAL and circulating specific IgG can be found in exposed subjects without active EAA, and it is not known what are the triggering factors needed to cause EAA in exposed subjects (Selman 1998, Rose 2000).

EAA is usually a benign disease as compared with CFA. If the offending antigen is avoided, the granulomas and inflammation resolve. However, with prolonged exposure, fibrosis and irreversible changes may evolve in the peripheral lung (Selman 1998).

2.2.4 NO in pulmonary physiology and pathophysiology

All three isoforms of NOS have been detected in the human lung (Hamid et al. 1993, Kobzik et al. 1993, Asano et al. 1994). Although iNOS expression is usually associated with inflammatory states, some "constitutive" iNOS expression seems to be present in the airway epithelium of healthy subjects (Saleh et al. 1998). This might reflect the continuous inhalation of irritant factors which induce iNOS expression, as in *ex vivo* cultures airway epithelial cells from healthy subjects soon lose iNOS expression (Guo et al. 1995).

NO and NO-releasing compounds relax bronchial smooth muscle in a cGMP-dependent manner and cause bronchodilatation (Di Maria et al. 2000). NO acts as an endogenous neurotransmitter of the so-called non-adrenergic non-cholinergic nerves (NANC-nerves) which relax smooth muscle in the bronchial tree (Belvisi et al. 1992). NO is an important endogenous molecule counteracting bronchoconstriction in the human airways. Inhalation of NOS inhibitor considerably increases bronchial responsiveness to inhaled bradykinin or methacholine in asthma (Sterk et al. 1999), whereas inhalation of a high NO concentration after such a provocation attenuates bronchoconstriction (Högman et al. 1993, Kacmarek et al. 1996).

Constitutive NO synthesis is an important factor maintaining microvascular integrity in the bronchial circulation. However, increased NO production in inflammation dilates the arterioles and increases blood flow to leaky post-capillary venules and thus exacerbates airway oedema (Bernareggi et al. 1997). NO is also an important vasodilating mediator in the pulmonary circulation, and inhaled NO in high concentrations can be used to improve pulmonary blood oxygenation and to treat pulmonary hypertension (Hurford et al. 2000).

NO has a dual role in bronchial mucus secretion. Low constitutive NO production

inhibits this process (Ramnarine et al. 1996), whereas increased NO production in inflammation enhances it (Adler et al. 1995, Barnes 1998). NO is thought also to regulate mucociliary clearance, as NOS inhibitors have been reported to reduce ciliary beat frequency after stimulation by bradykinin or substance P (Jain et al. 1993).

High levels of NO in inflammation are thought to have cytotoxic effects on the bronchial epithelium, and this might cause epithelial shedding found e.g. in asthma (Barnes 1998). These effects are likely to be mediated by peroxynitrite, which is formed by NO reacting with the superoxide produced in high amounts during inflammation.

2.2.5 NO in asthmatic inflammation

As compared with healthy subjects, increased iNOS expression has been reported in bronchial biopsies from asthmatic subjects (Hamid et al. 1993, Saleh et al. 1998, Redington et al. 2001). Furthermore, iNOS expression can be reduced by treatment with glucocorticoids (Saleh et al. 1998, Redington et al. 2001). iNOS expression in asthmatic airways has been shown in epithelial cells, macrophages, eosinophils, neutrophils, vascular endothelium and smooth muscle cells (Hamid et al. 1993, Saleh et al. 1998, ten Hacken et al. 2000, Redington et al. 2001). The increased amount of NO in the exhaled air of asthmatic subjects is largely produced by the iNOS pathway, as inhalation or an oral dose of selective iNOS inhibitors causes a significant reduction in exhaled NO level (Yates et al. 1996, Erin et al. 2002).

High NO production by iNOS is thought to have many detrimental effects in asthmatic airways, for example oedema formation by reduced vascular integrity, epithelial cell toxicity and increased hyperresponsiveness caused by peroxynitrite (Heiss et al. 1994, Curran 1996, Sadeghi-Hashjin et al. 1996).

NO has been found to inhibit proliferation of murine Th_1 lymphocytes and their secretion of IL-2 and IFN-ν. By contrast, no effect on Th_2 lymphocytes was evidenced (Taylor-Robinson et al. 1994). It has thus been suggested that NO plays a role in regulating the balance between Th_1 and Th_2 lymphocytes also in humans. By inhibiting the Th_1 cell response, NO would favour the Th_2 response and secretion of IL-4 and IL-5, further leading to IgE production and eosinophil recruitment into the airways (Barnes and Liew 1995). However, studies assessing Th_1 / Th_2 response in murine cells may not be conclusive for human lymphocytes, as there are considerable inter-species differences in the regulation of the immune response.

The role of NO in asthmatic inflammation and airway hyperresponsiveness has been studied in animal models of asthma using either NOS inhibitors or NOS knockout mice (Folkerts et al. 2001). The non-selective NOS inhibitor L-NMMA and the partially cNOS-selective L-NAME reduce experimentally induced airway hyperresponsiveness and eosinophilic airway inflammation in ovalbumin-induced asthma models in guinea pigs (Iijima et al. 1998), rats (Ferreira et al. 1998) and mice (Feder et al. 1997, Trifilieff et al. 2000), although contradictory results have also been presented (Mehta et al. 1997,

Tulic et al. 2000). Selective iNOS inhibitors have been used to determine the specific role of iNOS-derived NO in these ovalbumin-induced asthma models. Aminoguanidine reduced airway hyperresponsiveness in guinea pigs (Iijima et al. 1998) and eosinophilic airway inflammation in both rats (Tulic et al. 2000) and mice (Feder et al. 1997). The more selective iNOS inhibitors L-NIL and 1400W had no effect on eosinophilic inflammation in mice or rats but reduced airway hyperresponsiveness (Feder et al. 1997, Muijsers et al. 2001, Eynott et al. 2002).

Knockout mice of each NOS isoform have been studied in an ovalbumin-induced asthma model. Surprisingly, nNOS knockout mice showed lower airway responsiveness than wild-type controls (De Sanctis et al. 1999). iNOS knock-outs were reported to have either reduced or similar eosinophilic airway inflammation as in wild-type controls, but no change was found in airway responsiveness (De Sanctis et al. 1999, Xiong et al. 1999).

The conflicting results from these animal studies might reflect problems with the specificity of NOS inhibitors, or differences between the asthma models used. Although these animal studies give *in vivo* information on the role of different NOS isoforms, their interpretation in terms of human asthma is complicated.

In humans, inhalation of the non-selective NOS inhibitor L-NAME has been found to have no effect on allergen challenge-related airway obstruction either in the early or late asthmatic reaction (Taylor et al. 1998). A significant decrease in exhaled NO level was evidenced, as expected, but whether NOS inhibition caused changes in the recruitment of inflammatory cells was not measured.

Recent findings also link NO to the pathogenesis of asthma by showing a relation between certain nNOS and eNOS gene polymorphisms and asthma (Gao et al. 2000, Grasemann et al. 2000, Lee et al. 2000). Gao and colleagues studied variants of nNOS exon 2 and found that a 183 bp allele was significantly more frequently present in asthmatics than in controls. Grasemann and colleagues studied CA repeat polymorphism in the exon 29 of the nNOS gene, and found that in asthmatics the allele with 18 repeats was significantly less common whereas that with 17 repeats was significantly more common than in controls. However, the functional significance of these polymorphisms in either exon 2 or 29 is not known and their role in the pathogenesis of asthma cannot thus be addressed. A defect in nNOS-dependent bronchodilatory innervation could, in theory, be one mechanism predisposing to symptomatic asthma.

Lee and colleagues studied variation in exon 4 of eNOS and found two alleles of different lengths; allele a (573 bp) and allele b (604 bp). Genotype b/b was significantly more frequent in asthmatics than in controls (Lee et al. 2000). The b/b genotype has been shown to be associated with higher circulating NO metabolite levels than the b/a or a/a genotypes (Tsukada et al. 1998), but how this might affect the pathogenesis of asthma is not known.

2.2.6 NO and inflammation in CFA and EAA

In patients with CFA, iNOS expression has been shown by immunohistochemistry in airway and alveolar epithelial cells, macrophages, neutrophils, and in some cases also in lymphocytes, vascular endothelium and smooth muscle of airways and vessels. Also nitrotyrosine was shown in the same cells. Further, immunostaining for both iNOS and nitrotyrosine was higher in CFA patients with early to intermediate disease stage as compared with patients having end-stage fibrosis (Saleh et al. 1997). In patients with CFA or fibrosing alveolitis associated with systemic sclerosis (FASSc), activated fibroblasts in the early disease stage showed immunostaining for iNOS, whereas in late-stage fibrosis with less active fibroblasts, only little iNOS was found (Romanska et al. 2000b). Stimulation of human pulmonary fibroblasts with cytokines induces iNOS expression and increases the cell population, whereas the NOS inhibitor L-NMMA reverses the cytokine effect on cell count (Romanska et al. 2000a). iNOS expression and detrimental effects mediated by a high NO production rate seem to be associated with active inflammation and fibrogenesis of the disease process.

Intra-tracheal bleomycin administration is used to induce a model of pulmonary fibrosis in rodents. Inhibition of iNOS by aminoguanidine prevents fibrotic changes and reduces the pulmonary content of hydroxyproline in this model (de Rezende et al. 2000, Giri et al. 2000), suggesting that iNOS-dependent high NO production is detrimental in the pathogenesis of pulmonary fibrosis.

A recent interesting finding suggests a protective role for constitutive NO production in pulmonary fibrosis. In transgenic mice overexpressing eNOS, bleomycin administration causes significantly less fibrosis and hydroxyproline production than in wild-type mice. NOS inhibition by L-NAME abolishes the protecting effect of eNOS overexpression (Yoshimura et al. 2002). NO might thus have a dual role also in the pathogenesis of pulmonary fibrosis as in inflammation, constitutive NO production protects from fibrotic events whereas high iNOS-dependent NO production is detrimental.

There are no published papers assessing the role of NO in EAA, but one abstract reports increased levels of nitrate in the BAL fluid of patients with EAA (Lange et al. 2000). However, as iNOS has been detected by immunohistochemistry in granulomas in patients with sarcoidosis or pulmonary tuberculosis (Moodley et al. 1999), there is probably iNOS activation also in the granulomas in EAA, although the granulomas differ between these diseases.

2.3 Exhaled NO Measurement

Part of the NO produced in the lungs diffuses into the pulmonary air. In 1991, Gustafsson and colleagues reported that exhaled air of rabbits, guinea pigs and humans contains endogenously produced NO (Gustafsson et al. 1991). They found NO in exhaled air from all three species although the inhaled air was free of NO. NOS inhibitors administered to rabbits and guinea pigs reduced exhaled NO

concentrations, while administration of L-arginine increased the exhaled NO concentration back towards normal. Based on these results it was assumed that pulmonary diseases associated with altered NO production rate could be assessed by measuring the NO concentration in exhaled air.

This assumption was confirmed by two study groups, who reported that patients with asthmatic airway inflammation have increased exhaled NO concentrations as compared with healthy subjects (Alving et al. 1993, Persson et al. 1994). In 1994, Kharitonov and colleagues further reported that steroid-naive asthmatics exhibited an increased exhaled NO concentration, while asthmatics treated with inhaled steroids had normal concentrations (Kharitonov et al. 1994b).

In these early studies exhaled air was found to contain endogenously produced NO, whose concentration was increased in patients with untreated asthmatic airway inflammation, while anti-inflammatory drug treatment of the inflammation reduced the concentration back towards normal. These findings made exhaled NO measurement a promising new non-invasive method to assess airway inflammation in pulmonary diseases. Since then, great interest has been shown towards exhaled NO measurement in inflammatory lung diseases, and several hundred scientific papers have hitherto been published on the topic. Basic methodology, most important results and recent progress in exhaled NO measurement will be reviewed in this chapter.

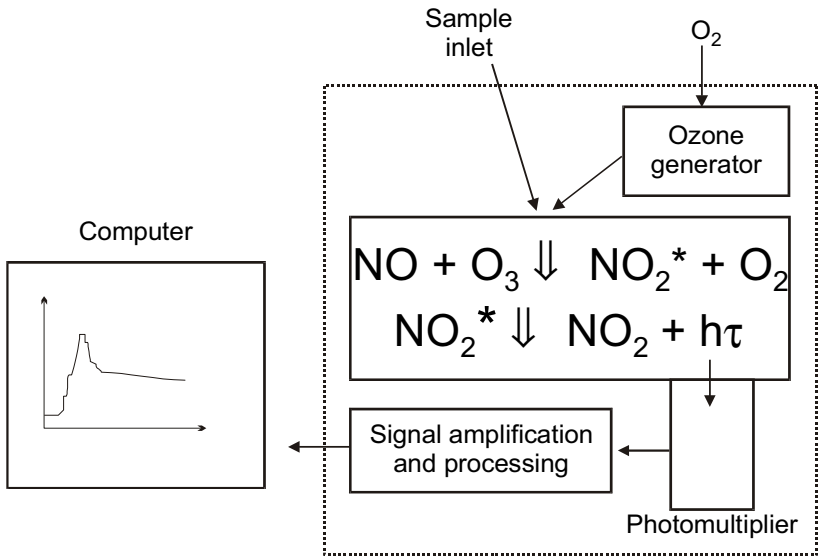

Figure 4. Schematic illustration of an ozone-chemiluminescence NO analyser. Red and infrared light is emitted as NO reacts with ozone. The NO concentration in the sample gas can be calculated by quantifying the amount of light emitted.

2.3.1 Basic methodology of exhaled NO measurement

NO analysis. The nitric oxide concentration in exhaled air is usually measured by the ozone-chemiluminescence method. This approach is based on the reaction between NO and ozone (O_3) forming nitrogen dioxide (NO_2), some of which is in excited state (NO_2^*). Red and infrared light (~ 640 – 3000 nm) is emitted as the excited form of nitrogen dioxide regains its stable ground state ($NO_2^* \Downarrow NO_2 + h\nu$) (Hampl et al. 1996). The amount of light can be quantified by a photomultiplier. In stable conditions, the amount of light emitted is proportional to the amount of NO in the specimen gas, making ozone-chemiluminescence a precise and sensitive method for measuring the NO content of exhaled air (Figure 4).

Exhalation manoeuvre. In the early studies, exhaled NO was measured during tidal breathing or uncontrolled slow vital capacity manoeuvres (Alving et al. 1993, Kharitonov et al. 1994a, Kharitonov et al. 1994b). During tidal breathing, the measured NO concentration varies considerably in the course of the breathing cycle. Changes in breathing pattern also cause variation in exhaled NO levels, and more repeatable manoeuvres were sought.

Real-time measurement of NO concentration during a single slow exhalation from total lung capacity is now preferred (Kharitonov et al. 1997a, American Thoracic Society 1999). The initial part of the NO curve representing the dead-space air is somewhat variable, but the end-expiratory plateau in the NO curve during a single exhalation is highly repeatable. A high initial peak in the curve may be caused by accumulation of NO in the airways during a breath-hold before exhalation or by a high NO level in inhaled air (high ambient NO level or inhalation through the nose where the NO production

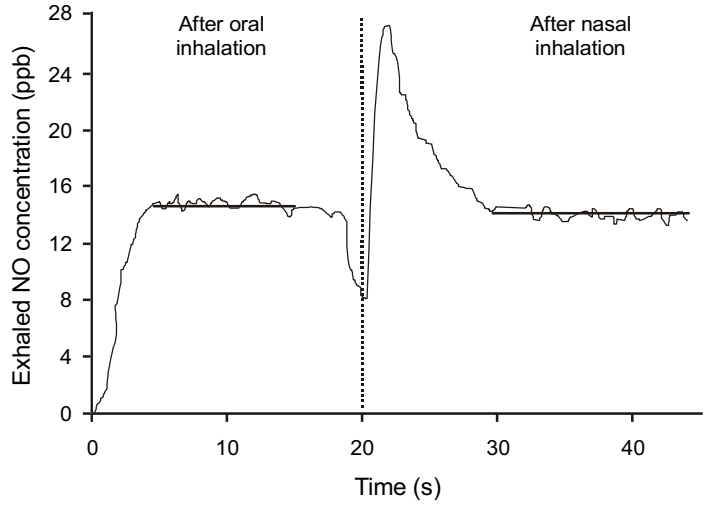

Figure 5. Exhaled NO concentration curves during single exhalations after oral inhalation and nasal inhalation. Inhalation of nasal air with high NO concentration causes an initial peak in the exhaled NO profile, but the end-expiratory plateau is not different from that after oral inhalation. Modified from American Thoracic Society (1999).

rate is high). However, even after such a peak the normal end-expiratory plateau representing the endogenous NO production in the lower airways is reached (Silkoff et al. 1997) (Figure 5).

With the single exhalation manoeuvre, certain important factors such as exhalation flow rate and discarding of nasal NO need to be taken into account (as discussed in greater detail below). In small children who are not able to control their breathing and cannot produce a long steady exhalation with a fixed flow rate, NO measurement has to be done during tidal breathing. Manually adjustable flow restrictors have been used in small children to fix the exhalation flow rate externally to the desired level during tidal breathing (Buchvald and Bisgaard 2001). This improves the repeatability of tidal breathing measurements by eliminating the variability in NO values caused by variation in exhalation flow rate. Off-line measurements with collection of exhaled air into a reservoir to be transported for later analysis allow NO measurement in subjects away from the actual NO analyser. Off-line measurements have a good correlation with on-line measurements, provided that the exhalation flow rate and sufficient exhalation pressure are strictly controlled in both methods (Kissoon et al. 2000, Kissoon et al. 2002).

Exhalation flow rate. The first exhaled NO measurements were carried out in patients breathing normal tidal breathing or during slow uncontrolled vital capacity manoeuvres. In later studies, the exhalation flow rate was found to have a major effect on the NO concentration in exhaled air (Byrnes et al. 1997, Högman et al. 1997, Silkoff et al. 1997). The exhaled NO concentration decreases with increasing flow rates, while the total NO output increases with increasing flow rates (Figure 6). This is attributed to the significant airway contribution to total exhaled NO concentration (Silkoff et al. 1997). With higher flow rates, alveolar air travels more rapidly through the bronchial tree, reducing the time available for alveolar gas to gain more NO from the bronchial mucosa. Variation in exhalation flow rate therefore diminishes the repeatability of exhaled NO measurement. If the exhalation flow rate is

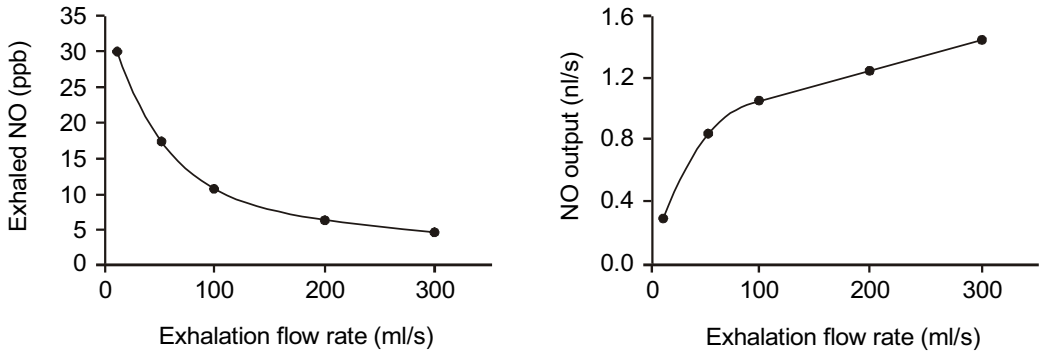

Figure 6. Exhaled NO concentration and NO output at exhalation flow rates of 10, 50, 100, 200 and 300 ml/s in a healthy child (Lehtimäki et al., unpublished).

adequately controlled, exhaled NO measurement is highly repeatable (Silkoff et al. 1997, Gabbay et al. 1998, Ekroos et al. 2000, Ekroos et al. 2002). ERS guidelines from 1997 recommend a constant flow rate from 10 to 15 l/min (167-250 ml/s) (Kharitonov et al. 1997a), while more recent guidelines by ERS and ATS recommend a flow rate of 50 ml/s (American Thoracic Society 1999, Baraldi and de Jongste 2002).

Mouth pressure. The mucosa in the nasal cavity and paranasal sinuses produces considerable amounts of NO (Lundberg et al. 1995) which accumulates in high concentrations in nasal air compared with air from the lower respiratory tract (Kimberly et al. 1996). When measuring exhaled NO concentration from the lower respiratory tract, contamination of the sample with nasal air should be avoided. A low positive mouth pressure (−4 cmH_2O) during exhalation is sufficient to close up the soft palate and hence separate the nasal cavity from the oral cavity (Kharitonov and Barnes 1997b, Silkoff et al. 1997) (Figure 7). This ensures that there is no leak of nasal air into the sample air from the lower respiratory tract, and nasal NO contamination can be avoided. An internal resistance in the exhalation circuit of the measurement device is therefore recommended to obtain mouth pressures from 5 to 20 cmH_2O during exhaled NO measurement (American Thoracic Society 1999).

NO in ambient air. Inhalation of NO-free air (< 5 ppb) is recommended in exhaled NO measurement (American Thoracic Society 1999), although it has been shown that even high levels of ambient NO (up to 150 ppb) (Piacentini et al. 1998) or inhalation of a gas mixture with a high NO concentration (up to 1 000 ppb) have no effect on the end-expiratory NO plateau (Silkoff et al. 1997). However, inhalation of air with a high NO concentration causes a high initial peak in the exhaled NO concentration curve (Silkoff et al. 1997).

Diet. NO may be produced in the oral cavity not only by NOS enzymes but also in a non-enzymatic reduction from nitrite. The

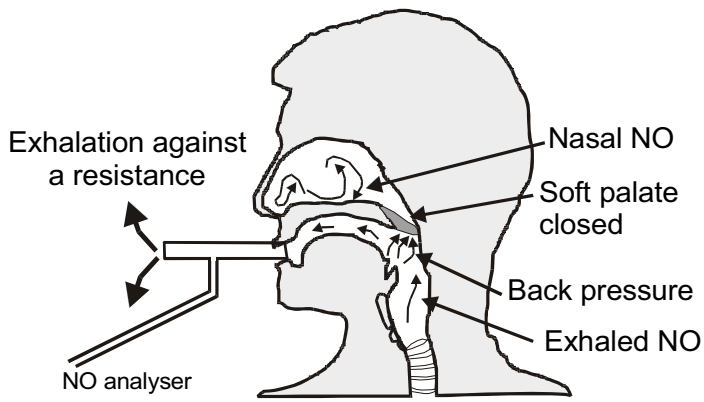

Figure 7. Exhalation against a resistance during NO measurement produces a low positive mouth pressure which closes up the soft palate and hence separates nasal air with high NO concentration from the air coming from the lower respiratory tract. Modified from Barnes (1998).

salivary glands excrete nitrate from the circulation into saliva, and bacteria in the oral cavity convert nitrate into nitrite. In an acidic oral environment nitrite is further reduced to form NO. Ingestion of potassium nitrate or a meal rich in nitrate prior to NO measurement has been shown to increase the exhaled NO level, whereas rinsing the mouth with anti-bacterial or basic solution reduces the level (Zetterquist et al. 1999, Olin et al. 2001). It is thus recommended that patients should refrain from eating and drinking 1 h prior to exhaled NO measurement (American Thoracic Society 1999), and rinsing of the mouth with basic solution may be used to reduce the non-enzymatic oral NO output.

Pulmonary function testing. Repeated forced vital capacity manoeuvres with spirometry have been shown to lower exhaled NO levels, whereas inhaled η_2-agonists after spirometry increase the level (Silkoff et al. 1999). Exhaled NO measurement should thus be performed prior to any possible measures of lung function.

Smoking. Cigarette smoking has been found to reduce exhaled NO levels, while discontinuation of smoking increases exhaled NO levels gradually towards normal levels (Persson et al. 1994, Kharitonov et al. 1995b, Robbins et al. 1996, Robbins et al. 1997). As cigarette smoke contains high levels of NO (Borland and Higenbottam 1987), it has been suggested that endogenous pulmonary NO production is reduced in smokers due to the downregulation of NOS activity by exogenous NO (Assreuy et al. 1993, Kharitonov et al. 1995b). Smoking has also been shown to reduce the bioavailability of BH_4, an important cofactor in NO synthesis (Higman et al. 1996, Heitzer et al. 2000, Ueda et al. 2000). NO synthesis might therefore be reduced in smokers regardless of normal or even enhanced NOS expression. However, exhaled breath condensate from active smokers contains normal levels of NO metabolites such as nitrite and nitrate despite the lower level of exhaled NO (Balint et al. 2001). This suggests that the lower NO level in exhaled air of smokers might not be explained by decreased NO production rate but rather by increased metabolic consumption of NO (e.g. reacting with superoxide to form peroxynitrite).

2.3.2 International guidelines on exhaled NO measurement

As discussed above, a number of important technical factors need to be taken into account while measuring the NO concentration in exhaled air. Most important of these are the inverse relation between exhalation flow rate and NO concentration in exhaled air, and the need for a positive mouth pressure closing up the soft palate to avoid nasal contamination. Before these factors were strictly controlled in earlier studies, there was significant variation between study groups in measurement technique, causing conflicting results. To overcome this problem, international guidelines have been published to standardise the measurement technique. Nowadays exhaled NO concentration is recommended to be measured during a single breath at a constant exhalation flow rate of 50 ml/s against a low mouth pressure of 5 – 20 cmH_2O (American Thoracic

Society 1999, Baraldi and de Jongste 2002). An abnormally high exhaled NO concentration is interpreted as a sign of ongoing inflammation in the lower respiratory tract.

2.4 Exhaled NO Concentration in Inflammatory Lung Diseases

The concentration of NO in exhaled air has been assessed in many different inflammatory lung conditions. In most studies, the exhaled NO concentration has been measured at a single exhalation flow rate. Early studies used uncontrolled slow exhalations or tidal breathing, but the recommended single-breath method with counterpressure and fixed exhalation flow rate is nowadays the approach mostly used. The latest progress in exhaled NO measurement technique is the use of multiple different exhalation flow rates to differentiate between alveolar and bronchial NO output. The theory underlying this method will be reviewed in the next section (2.5 Mathematical Modelling of Pulmonary NO Dynamics). The most important results on exhaled NO measurement at a single exhalation flow rate in asthma and alveolitis are reviewed here, other lung diseases being only briefly mentioned.

2.4.1 Exhaled NO in asthma

Asthma was the first lung disease in which exhaled NO levels were reported. An increased exhaled NO concentration was found during tidal breathing in patients with asthma in 1993 (Alving et al. 1993), and soon after, asthmatic patients treated with inhaled glucocorticoids were reported to have lower peak exhaled NO values than steroid-naïve patients (Kharitonov et al. 1994b). Increased levels of exhaled NO have since been found in many studies utilising the single-breath technique in both adults and children with asthma (Horvath et al. 1998, Mattes et al. 1999, Silkoff 2000, Kharitonov and Barnes 2001). The increased exhaled NO concentration in asthma has been attributed to enhanced iNOS expression in the airway epithelium and inflammatory cells in asthmatic airways (Hamid et al. 1993, Saleh et al. 1998). A considerable number of papers have been published concerning exhaled NO measurement in asthma, and it is the most widely studied pulmonary disease in terms of exhaled NO measurement.

Atopy, asthma and exhaled NO. Exhaled NO levels are higher in atopic than in non-atopic asthma (Frank et al. 1998, Ho et al. 2000a). Exhaled NO levels among asthmatics are correlated with the number of positive skin prick tests (Ho et al. 2000a), blood eosinophil count (Silvestri et al. 1999) and serum levels of IgE (Simpson et al. 1999, Ho et al. 2000a). The reason for the higher NO levels in atopic versus non-atopic asthmatics is not known, but the finding might be related to different inflammatory mechanisms in these patient groups. Increased exhaled NO levels have been found even in atopic adults (Horvath and Barnes 1999) and children (Franklin et al. 1999) without symptoms of asthma. This might be related to subclinical airway inflammation in the lower respiratory tract without detectable changes in airway function.

A challenge test with specific allergens in atopic asthmatics does not affect the exhaled NO concentration during early asthmatic reaction but increases exhaled NO levels during late asthmatic reaction in association with decreased FEV_1 (Kharitonov et al. 1995a, Piipari et al. 2002). In atopic asthmatics, the exhaled NO concentration increases in association with enhanced airway inflammation during a low-dose allergen exposure even in the absence of changes in lung function (de Kluijver et al. 2002). Likewise naturally occurring allergen exposure increases exhaled NO levels. Atopic asthmatics with significant indoor exposure to their specific allergens have higher exhaled NO levels than unexposed atopic asthmatics (Simpson et al. 1999). In asthmatic children sensitised to the house-dust mite, moving to a residential house with low levels of mite allergen lowers exhaled NO levels (Piacentini et al. 1999), while moving back to high allergen exposure increases the concentration back to high levels (Piacentini et al. 1999, Piacentini et al. 2000). The increase in exhaled NO upon returning to high allergen exposure can be avoided by inhaled glucocorticoid treatment (Piacentini et al. 2000). Atopic asthmatics have shown higher exhaled NO levels associated with increased symptoms during the pollen season than outside the season, even though no change was evidenced in FEV_1 during pollen exposure (Baraldi et al. 1999). These findings support the role of exhaled NO measurement in assessing the severity of allergic airway inflammation in atopic asthmatics.

Exhaled NO and other markers of asthma. Either a weak (Sippel et al. 2000) or no correlation (Horvath et al. 1998, Jatakanon et al. 1998, Ho et al. 2000a) has been found between exhaled NO concentration and spirometric parameters of lung function (FEV_1) in asthma. Although the exhaled NO concentration does not appear to reflect fixed airflow obstruction, it is positively correlated with diurnal PEF variation (Al-Ali et al. 1998, Lim et al. 1999) and with bronchial hyperresponsiveness to histamine (Dupont et al. 1998) or methacholine (Lim et al. 1999). However, while exhaled NO levels decrease after treatment with inhaled glucocorticoids, the correlation between exhaled NO and bronchial responsiveness is also disturbed (Dupont et al. 1998, Lim et al. 1999). This might be explained by a more rapid response of exhaled NO to anti-inflammatory treatment, or that bronchial hyperresponsiveness cannot always be totally abolished by anti-inflammatory treatment if airway remodelling has occurred. Also the significant improvement in FEV_1 after inhaled η_2-agonist is related to a higher exhaled NO concentration (Sippel et al. 2000). The baseline exhaled NO level also correlates with the degree of exercise-induced bronchoconstriction (Scollo et al. 2000, Terada et al. 2001). It would thus appear that exhaled NO concentration is not a good marker of fixed airway obstruction, but it is related to bronchial hyperresponsiveness and reversibility or variability in airflow obstruction associated with inflammatory activity.

Exhaled NO levels have also been found to correlate with more direct measures of asthmatic airway inflammation such as the number of eosinophils in sputum (Jatakanon et al. 1998, Chan-Yeung et al. 1999, Mattes et al. 1999) and BAL fluid (Lim et al. 1999,

Warke et al. 2002). A correlation has also been found between exhaled NO levels and indices of eosinophilic inflammation in bronchial mucosal biopsies (Payne et al. 2001, van den Toorn et al. 2001), but not in all studies (Lim et al. 2000).

Drug treatment and exhaled NO in asthma. Inhaled glucocorticoids in patients with asthma reduce the exhaled NO concentration while alleviating asthmatic airway inflammation (Kharitonov et al. 1996a, Lim et al. 1999, van Rensen et al. 1999). Also oral and parenteral glucocorticoids lower exhaled NO levels in asthma (Massaro et al. 1995, Baraldi et al. 1997, Nelson et al. 1997). If inhaled glucocorticoids are withdrawn after a short treatment period, exhaled NO rises back to pre-treatment level, and the reduction in exhaled NO level can be repeated in a similar manner after re-introducing inhaled steroids (Silkoff et al. 2001). The decrease in exhaled NO concentration after inhaled glucocorticoids is dose-dependent, such that a higher dose of inhaled glucocorticoids reduces the exhaled NO concentration more and faster than a lower dose (Jatakanon et al. 1999a, Silkoff et al. 2001, Jones et al. 2002, Kharitonov et al. 2002). Exhaled NO concentration is sensitive to the anti-inflammatory effect of inhaled glucocorticoids even in low doses (Jatakanon et al. 1999a, Silkoff et al. 2001). Exhaled NO level also responds to inhaled glucocorticoids very rapidly, showing a significant decrease already 6 hours after introducing a high dose of inhaled budesonide (Tsai et al. 2001). The reducing effect of inhaled glucocorticoids on exhaled NO can be explained by reduced iNOS expression in the asthmatic airways following the treatment (Saleh et al. 1998). Glucocorticoids may reduce iNOS expression directly by interfering with the transcription and translation of iNOS (inhibition of NF-ρB or destabilising of mRNA), or indirectly by reducing the production of the pro-inflammatory cytokines (e.g. IL-1, TNF-ζ) responsible for induction of iNOS expression.

Treatment with leukotriene receptor antagonists has been reported to reduce exhaled NO concentrations (Bisgaard et al. 1999, Bratton et al. 1999, Wilson et al. 2001), although this has not been the case in all studies (Yamauchi et al. 2001, Dempsey et al. 2002). The potency of antileukotrienes to reduce exhaled NO levels seems to be weaker than that of inhaled glucocorticoids (Bisgaard et al. 1999). Neither low-dose theophylline (Lim et al. 2001) nor nedocromil has any effect on exhaled NO in patients with asthma (Carra et al. 2001). The anti-inflammatory effect of these agents is also weaker than that of steroids.

Inhaled η_2-agonists have no long term effect on the exhaled NO concentration (Yates et al. 1997, Fuglsang et al. 1998, Aziz et al. 2000). This is comprehensible, since these drugs have no major anti-inflammatory effect. However, while bronchoconstriction after several different types of stimuli slightly reduces exhaled NO levels (de Gouw et al. 1998, Scollo et al. 2000, Terada et al. 2001), inhaled η_2-agonists after challenge increase exhaled NO levels with increasing FEV_1 (Ho et al. 2000b). This is very likely due to enhanced bronchial diffusion of NO after dilating the airways and thus increasing the mucosal surface area available for this diffusion.

Exhaled NO and asthma control. Reduction of dose or discontinuation of treatment with inhaled glucocorticoids increases both exhaled NO concentration and asthmatic symptoms, while re-introduction of the previous dose reduces the exhaled NO level and symptoms (Kharitonov et al. 1996b, Silkoff et al. 2001). Higher exhaled NO levels are associated with recent asthmatic symptoms and with the need for rescue η_2-agonists (Stirling et al. 1998, Artlich et al. 1999, Sippel et al. 2000). Exhaled NO measurement might thus serve to indicate whether a given dose of inhaled glucocorticoids is sufficient.

Increased exhaled NO levels in asthmatics on regular inhaled glucocorticoids can be used to predict the beneficial effect of adding oral prednisolone to their conventional therapy. Patients with an increased exhaled NO level while on inhaled glucocorticoids have shown improved lung function after adding oral prednisolone 30 mg/day for two weeks to their therapy. Patients with low exhaled NO concentration during their normal inhaled glucocorticoid treatment did not benefit from adding oral steroids (Little et al. 2000). This suggests that exhaled NO measurement could be used to find the optimal steroid dose.

Recent results suggest that the exhaled NO concentration can also be used to predict loss of asthma control after discontinuation of drug treatment. Both a high exhaled NO level before withdrawal of medication and a significant increase in exhaled NO after the withdrawal can be used to predict loss of asthma control (Jones et al. 2001). Jones and colleagues found that loss of asthma control could be predicted by exhaled NO measurement as reliably as with measures of sputum eosinophils or bronchial responsiveness. However, exhaled NO level before discontinuing inhaled glucocorticoids has not proved useful in predicting loss of asthma control in all studies (Leuppi et al. 2001).

2.4.2 Exhaled NO in CFA and EAA

Only a few studies have assessed exhaled NO concentrations in patients with fibrosing or allergic alveolitis. A slightly increased concentration has been found in patients with CFA (Montuschi et al. 1998, Paredi et al. 1999). Exhaled NO in CFA correlates positively with the BAL fluid level of 8-isoprostane, a marker of oxidative stress (Montuschi et al. 1998). In patients with fibrosing alveolitis associated with systemic sclerosis, exhaled NO levels are increased (Rolla et al. 2000) especially in subjects with signs of active inflammation in BAL fluid (Paredi et al. 1999). These findings are in line with those of enhanced iNOS expression in the alveolar epithelium and inflammatory cells in patients with an active inflammatory process of CFA, as compared with low iNOS expression in patients with end-stage disease associated with honeycomb fibrosis (Saleh et al. 1997).

No peer-reviewed data on exhaled NO levels in patients with extrinsic allergic alveolitis have been published, but some abstracts report either increased (Lange et al. 2000) or normal (Boyd et al. 2000) exhaled NO levels in these subjects.

2.4.3 Exhaled NO in other pulmonary diseases

Several studies have assessed exhaled NO levels in COPD, with conflicting results. Normal or slightly increased levels of exhaled NO have been reported (Kanazawa et al. 1998, Maziak et al. 1998, Rutgers et al. 1998, Ichinose et al. 2000). The discrepancies very likely reflect differences in the methodology used and different phenotypes of patients with this heterogeneous disease. Although increased pulmonary NO production has been reported in COPD (increased iNOS expression, nitrotyrosine and nitrite/nitrate in sputum), exhaled NO levels are normal or only slightly increased (Kanazawa et al. 1998, Ichinose et al. 2000). This might be explained by an increased production of superoxide which reacts with NO and thus scavenges NO from diffusion into the gas phase. Increased exhaled NO levels are associated with unstable COPD (Maziak et al. 1998) and with reversible airflow obstruction and eosinophilic inflammation, which predict a favourable response to steroid treatment (Chanez et al. 1997, Papi et al. 2000). Exhaled NO measurement might thus aid in distinguishing patients who are likely to benefit from inhaled glucocorticoids.

Increased exhaled NO levels also have been found in other inflammatory airway diseases like sarcoidosis, bronchiectasis and pneumonia (Kharitonov et al. 1995c, Moodley et al. 1999, Adrie et al. 2001). Decreased exhaled NO levels are found in cystic fibrosis and primary ciliary dyskinesia (Grasemann et al. 1997, Loukides et al. 1998, Thomas et al. 2000, Kharitonov and Barnes 2001). In cystic fibrosis, the low exhaled NO values might be due to low iNOS expression or a high reaction rate of NO within the airways (Kharitonov and Barnes 2001, Morrissey et al. 2002).

2.5 Mathematical Modelling of Pulmonary NO Dynamics

Mathematical models of pulmonary NO dynamics have been developed to allow more sophisticated interpretation of exhaled NO measurements. In 1997, Hyde and colleagues presented a model describing NO dynamics in the alveolar region (Hyde et al. 1997). They assumed that NO produced in alveolar tissue diffuses into alveolar air at a certain rate ($J_{NO,Alv}$), causing NO to accumulate in alveolar air up to a certain concentration (C_{Alv}). NO in alveolar air can either be exhaled or it diffuses into the pulmonary blood circulation and is scavenged by haemoglobin. The diffusion rate of alveolar NO to the pulmonary capillaries is a product of the alveolar diffusing capacity of NO ($D_{NO,Alv}$) and alveolar NO concentration. After inhalation of air with a low NO concentration, NO from the alveolar tissue diffuses into the alveolar air and a steady-state dynamic equilibrium between tissue and gas phase is soon achieved. During the steady state, the diffusion of NO into the capillaries equals the diffusion of NO from the tissue to luminal air (Hyde et al. 1997) (Figure 8), giving:

$$C_{Alv} \Delta D_{NO,Alv} = J_{NO,Alv} \quad (1)$$

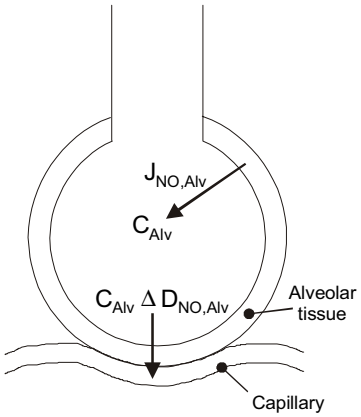

Figure 8. Model of pulmonary NO dynamics by Hyde et al. (1997). NO produced in alveolar tissue diffuses to alveolar air ($J_{NO,Alv}$), causing a certain concentration of NO in the alveolar air (C_{Alv}). The diffusion of NO from alveolar air to the pulmonary circulation equals the product of C_{Alv} and the alveolar diffusing capacity of NO ($D_{NO,Alv}$).

C_{Alv} can be assessed by measuring the end-exhalation NO plateau at a high exhalation flow rate (minimal airway contribution to exhaled NO concentration), and $D_{NO,Alv}$ can be measured using NO as the tracer gas in determining pulmonary diffusing capacity. With equation (1), $J_{NO,Alv}$ can be calculated based on these measurements, allowing approximation of alveolar NO production.

Experimental studies showing an inverse relation between exhaled NO concentration and exhalation flow rate led to the conclusion that a significant portion of NO in exhaled air originates from the conducting airways (Högman et al. 1997, Silkoff et al. 1997). The model by Hyde and colleagues described above considered only the alveolar region as a possible source of NO and neglected conducting airways.

In 1998, Tsoukias and George published a two-compartment model of pulmonary NO exchange dynamics which takes into account both the alveolar region and conducting airways (Tsoukias and George 1998). In the model, the lung is divided into two separate compartments, namely the alveolar and the bronchial compartments. The flexible or expansile alveolar compartment represents respiratory bronchioles and the alveolar region (generations 18 and beyond according to Weibel (1963)) where gas diffusion between alveolar air and pulmonary circulation takes place. The bronchial compartment is a single rigid cylindrical tube representing conducting airways larger than respiratory bronchioles (from trachea to generation 17 according to Weibel (1963)). Both compartments are surrounded by tissue layers capable of producing NO (alveolar membrane and bronchial mucosa, respectively). Both tissue layers, in turn, are surrounded by blood representing the bronchial circulation in the bronchial compartment and the pulmonary circulation in the alveolar compartment (Figure 9). NO produced in the tissue layers can follow one of the following paths: 1) consumption through reactions within the tissue, 2) diffusion into the surrounding blood circulation where NO is rapidly scavenged by haemoglobin, or 3) diffusion into the luminal air (alveolar or bronchial).

In both compartments there is a net diffusion of NO between tissue and gas phases, and the direction of the flux depends on the relative concentrations of NO in the tissue and gas phases. Alveolar air contains a certain concentration of NO (C_{Alv}) depending on alveolar NO dynamics as in the above-described model by Hyde and colleagues. The final NO concentration in exhaled air is dependent on two

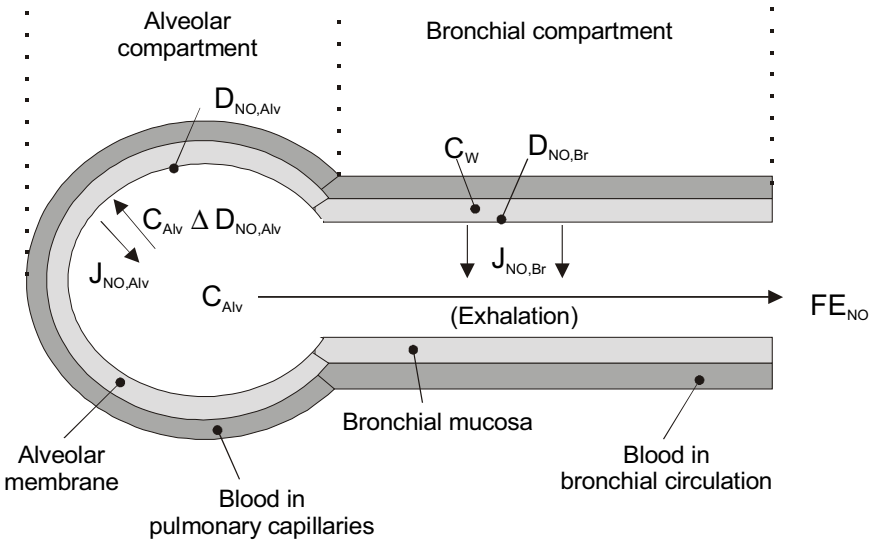

Figure 9. Schematic illustration of the two-compartment model of pulmonary NO dynamics (Tsoukias and George 1998, Silkoff et al. 2000). See text for details.

mechanisms: 1) NO concentration in alveolar air, and 2) conditioning of alveolar air while it travels through bronchial compartment during exhalation.

Accumulation of NO from the bronchial wall to exhaled air while it travels through bronchial tree can be modelled by dividing the bronchial compartment into infinitely short differential units. Upon entry to the bronchial compartment, the luminal air NO concentration equals the alveolar NO concentration. In the first unit NO diffuses from the bronchial wall to the luminal air, and at the entry to the second unit the luminal NO concentration equals the alveolar NO concentration + NO diffused in the first unit. Upon entry to the third unit the luminal NO concentration equals the alveolar NO concentration + NO diffused to luminal air in the first two units, and so forth. In every differential unit the diffusion of NO from bronchial wall to luminal air is driven by the NO concentration gradient between these two. The diffusion rate is determined by the bronchial diffusing capacity of NO ($D_{NO,Br}$). Conditioning of alveolar air in the bronchial compartment thus depends on the transit time of the alveolar air through conducting airways during exhalation (inverse to exhalation flow rate), the airway wall NO concentration (C_W), and the bronchial diffusing capacity of NO ($D_{NO,Br}$).

By formulating a differential equation based on the two-compartment model, the final NO concentration in exhaled air (FE_{NO}) can be expressed as a function of alveolar NO concentration (C_{Alv}), bronchial wall NO concentration (C_W), bronchial diffusing capacity of NO ($D_{NO,Br}$) and exhalation flow rate ($\dot{V}$), as follows (Tsoukias and George 1998, Högman et al. 2000, Silkoff et al. 2000):

$$FE_{NO} \mid C_W \left(4 e^{4\frac{D_{NO,Br}}{\dot{V}}} \right) 2 C_{Alv} \Delta e^{4\frac{D_{NO,Br}}{\dot{V}}} \quad (2)$$

As the total NO output from the lower respiratory tract is a product of exhaled NO concentration and exhalation flow rate ($\dot{V}_{NO} = FE_{NO} \cdot \dot{V}$), the total NO output can be written based on equation (2) as follows:

$$\dot{V}_{NO} = \left[C_W \left(1 - e^{-\frac{D_{NO,Br}}{\dot{V}}}\right) + 2\, C_{Alv} \cdot e^{-\frac{D_{NO,Br}}{\dot{V}}} \right] \cdot \dot{V} \quad (3)$$

These fundamental equations of the two-compartment model are able to simulate the experimentally demonstrated inverse relationship between FE_{NO} and $\dot{V}$, and the positive relationship between total NO output and $\dot{V}$ (Figure 10). After measuring the exhaled NO concentration (FE_{NO}) at several different known flow rates ($\dot{V}$), the unknown parameters (C_{Alv}, C_W and $D_{NO,Br}$) can be solved using the basic equations. This allows separate assessment of alveolar and bronchial NO dynamics.

At sufficiently high exhalation flow rates, when $\dot{V} \gg D_{NO,Br}$, the exponential function can be substituted with its approximate $e^{-\frac{D_{NO,Br}}{\dot{V}}} \approx 1 - \frac{D_{NO,Br}}{\dot{V}}$ (Högman et al. 2000). Equation (3) is then rearranged to give:

$$\dot{V}_{NO} = C_{Alv} \cdot \dot{V} + (C_W - C_{Alv}) D_{NO,Br} \quad (4)$$

Equation (4) presents NO output as a sum of two factors, namely NO convected from the alveolar compartment ($C_{Alv} \cdot \dot{V}$) and NO diffused from the bronchial wall to the luminal air ($(C_W - C_{Alv})D_{NO,Br}$). The latter term describes the theoretical maximum bronchial NO flux ($J_{NO,Br}$) reached at an infinitely high exhalation flow rate when the bronchial luminal NO concentration does not rise above the alveolar NO concentration. By introducing $J_{NO,Br}$ into equation (4), it can be rearranged to give:

$$\dot{V}_{NO} = C_{Alv} \cdot \dot{V} + J_{NO,Br} \quad (5)$$

Equation (5) presents $\dot{V}_{NO}$ as a linear function of $\dot{V}$, where C_{Alv} is the slope of the line and $J_{NO,Br}$ is the intercept between the line and the y-axis. C_{Alv} and $J_{NO,Br}$ can thus

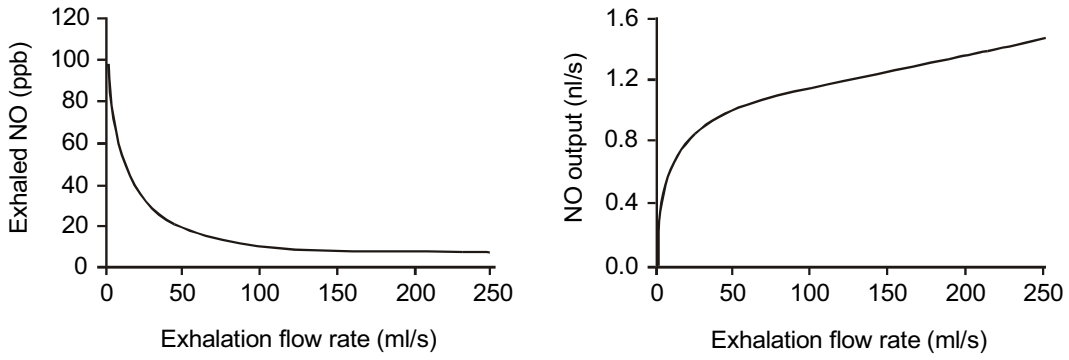

Figure 10. Simulation of exhaled NO concentration and NO output as functions of exhalation flow rate by the two-compartment model. Exhaled NO concentration and NO output are calculated according to equations 2 and 3 based on arbitrary values C_{Alv} = 2 ppb, C_W = 100 ppb and $D_{NO,Br}$ = 10 pl/s/ppb.

be solved by means of a regression line. After measuring the exhaled NO concentration at several different exhalation flow rates, $\dot{V}_{NO}$ is calculated for each $\dot{V}$ used. $\dot{V}_{NO}$ is then plotted against $\dot{V}$ and a regression line is set. The slope of the regression line is an approximate of C_{Alv} and the intercept is an approximate of $J_{NO,Br}$ (Tsoukias and George 1998, Högman et al. 2000) (Figure 11). Tsoukias and colleagues have further suggested that the parameters for alveolar and bronchial NO dynamics could be determined by measuring exhaled NO at a single exhalation with a dynamically changing exhalation flow rate instead of using several exhalations at different constant flow rates (Tsoukias et al. 2001b). This method, however, requires more complicated mathematics.

As shown in equation (1), the alveolar NO concentration can be increased by two factors; either enhanced NO production in alveolar tissue increases the diffusion rate of NO from alveolar tissue to alveolar air (increased $J_{NO,Alv}$), or an inflammatory / fibrosing process thickens the alveolar membrane and reduces the diffusing capacity of NO from alveolar air to pulmonary capillaries (decreased $D_{NO,Alv}$). Assessment of C_{Alv} and measurement of $D_{NO,Alv}$ would allow calculation of $J_{NO,Alv}$ to approximate the alveolar tissue NO production rate. As the pulmonary diffusing capacity of NO ($D_{NO,Alv}$) is approximately $4\,\Delta DL_{CO}$ (Borland and Higenbottam 1989), equation (1) can be rearranged to give

$$J_{NO,Alv} = C_{Alv}\,\Delta 4\,\Delta DL_{CO} \qquad (6)$$

Equation (6) allows calculation of $J_{NO,Alv}$ based on C_{Alv} and measurement of DL_{CO}, which is a routine test in pulmonary function laboratories. The alveolar NO production rate can thereby be assessed without measuring $D_{NO,Alv}$, which is not normally available.

The mathematical models above might help us better to understand the complicated dynamics of NO in the lungs, and thus hold a promise of more accurate interpretation of the results of exhaled NO measurement. If the exhaled NO concentration is measured only at a single exhalation flow rate, as recommended by current guidelines (Kharitonov et al. 1997a, American Thoracic Society 1999, Baraldi and de Jongste 2002), the presence of pulmonary inflammation can be detected based on an increased NO concentration in exhaled air. However, this method cannot be used to differentiate between alveolar and bronchial sources of NO, nor to differentiate between alveolar

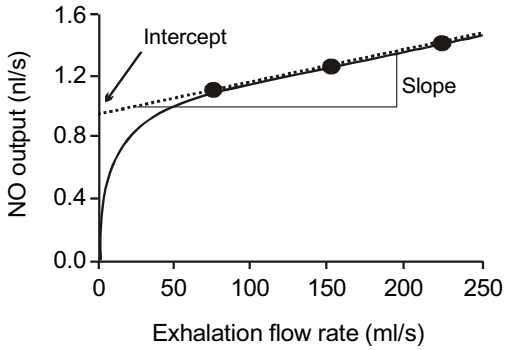

Figure 11. Plot of three NO output measurements (&) against flow rate. At higher flow rates (>50 ml/s), where NO output (solid curve) is almost linear, the bronchial NO flux approaches its theoretical maximum and can be considered constant. The slope and intercept of a regression line (dotted line) between NO output and flow rate are approximates of bronchial NO flux and alveolar NO concentration, respectively (Tsoukias and George 1998).

and bronchial inflammation. The mathematical models suggest that we could separate between alveolar and bronchial inflammation by measuring the exhaled NO concentration at multiple exhalation flow rates and then calculating the alveolar and bronchial contributions to the exhaled NO concentration. The aim of the present work was to test this possibility in practice. When this project was started, there were only theoretical publications concerning the possibility of differentiating between alveolar and bronchial inflammation by exhaled NO measurement. Other study groups have since published their results on applying the two-compartment model in patients with lung diseases. These results will be discussed in connection with the results of the present study in the Discussion.

3. AIMS OF THE STUDY

It was hypothesised that calculation of alveolar NO concentration and bronchial NO flux by the mathematical model of pulmonary NO dynamics might serve to differentiate between the alveolar and bronchial components of pulmonary inflammation. The aim of the present study was to test this hypothesis and to study the clinical applicability of the two-compartment model in assessing alveolar and bronchial NO output and inflammation in asthma and alveolitis. The detailed aims were:

1. to study whether calculation of alveolar and bronchial NO parameters based on exhaled NO measurement at multiple flow rates can be used to differentiate between alveolar and bronchial inflammation. (I and II)

2. to study whether alveolar and bronchial NO output are related to other markers of inflammation and disease severity in asthma and alveolitis. (II)

3. to study the effect of anti-inflammatory treatment on alveolar and bronchial NO output in alveolitis and asthma. (II and III)

4. to study whether alveolar and bronchial NO output are related to nocturnal symptoms among asthmatic patients. (IV)

5. to study whether alveolar or bronchial NO output is altered in patients showing symptoms of asthma but normal lung function (asthma-like syndrome). (V)

4. SUBJECTS AND METHODS

4.1 Subjects and Study Protocols

4.1.1 Two-compartment model in alveolar and bronchial inflammation (I, II)

To test whether the two-compartment model could be used to differentiate between alveolar and bronchial components in pulmonary inflammation, exhaled NO was measured at multiple exhalation flow rates in 40 patients with asthma, 17 patients with alveolitis and 57 healthy age-matched (∂ 5 years) and sex-matched controls. Alveolar and bronchial NO output was calculated for each subject. The correlation of alveolar and bronchial NO output to lung function, bronchial responsiveness and several inflammatory markers in serum and urine was studied in asthma. In alveolitis, the correlation of NO parameters to lung volumes and pulmonary diffusing capacity was studied.

To find patients with newly diagnosed asthma, 65 non-smoking steroid-naïve patients with asthmatic symptoms, who were referred for diagnostic evaluation from primary health care units to the Department of Respiratory Medicine at Tampere University Hospital, were recruited. The diagnostic investigations included thorough medical history, chest X-ray, physical examination, spirometry, methacholine challenge test and two-week PEF monitoring. Atopy was tested for by skin-prick test. An exercise test was performed if considered necessary based on patients' medical history. The diagnosis of asthma was based on symptoms and reversible or variable airway obstruction (η_2-agonist-induced increase in FEV_1 >12% and >200 ml, or η_2-agonist-induced increase in PEF or FVC >15%, or diurnal variation in PEF > 20% on three days during two weeks, or an exercise-induced decrease in FEV_1 > 15%) (American Thoracic Society 1991, National Heart Lung and Blood Institute 1997). Forty of the 65 patients met the diagnostic criteria for asthma and two had other disease causing their symptoms (one with gastro-oesophageal reflux and one with COPD); 23 patients had symptoms of asthma but did not fulfil the diagnostic criteria for asthma, and no other diseases causing their symptoms were found in clinical investigations.

Patients with alveolitis were also recruited from the Department of Respiratory Medicine at Tampere University Hospital. Their lung function was measured (spirometry and diffusing capacity) and plain radiograph or high resolution computed tomography used for radiological assessment of their disease. Seven patients were diagnosed with EAA (Terho 1986) and ten with CFA (American Thoracic Society and European Respiratory Society 2000). All EAA patients were farmers. Six of them were predisposed to mouldy hay and one to mouldy wood chips. Three of the patients with CFA had a biopsy-proven diagnosis of usual interstitial pneumonia (UIP), and the others had a clinical presentation typical of UIP. Five of the patients with alveolitis were already on anti-inflammatory medication when recruited into the study.

4.1.2 Effect of treatment on alveolar and bronchial NO output in asthma and alveolitis (II, III)

To study the effect of anti-inflammatory medication on alveolar and bronchial NO output in asthma, 16 asthmatic patients were treated with inhaled fluticasone (Flixotide Diskus, GlaxoWellcome, Ware, UK) for eight weeks (500 σg bid for weeks 1-4, 250 σg bid for weeks 5-8). Spirometry and exhaled NO were measured and asthma symptoms assessed before treatment and at weekly control visits during the eight-week treatment period. Inflammatory markers in blood and urine were measured before and after treatment.

To study the effect of anti-inflammatory treatment on alveolar and bronchial NO output in alveolitis, pulmonary diffusing capacity and exhaled NO were measured before and after two months of drug treatment and/or allergen avoidance in 7 patients with alveolitis. Five of the patients had EAA and 2 had CFA. None was on anti-inflammatory medication prior to the study. The intervention included either allergen avoidance (three patients with EAA) or allergen avoidance combined with oral prednisolone (2 patients with EAA), or oral prednisolone with or without azathioprine (2 patients with CFA).

4.1.3 Relation of alveolar and bronchial NO output to nocturnal symptoms of asthma (IV)

As a part of a written symptom questionnaire, nocturnal asthma symptoms

TABLE 1. Characteristics of patient groups in studies II - V.

Study	Group	N	Males (N)	Age (yrs)	FEV_1 (% pred)	$PD_{20}FEV_1$ <2.6 mg (N)	VC (% pred)	DL_{CO} (% pred)
II	Alveolitis	17	10	58 ∂ 3	-	-	76 ∂#4	52 ∂#3
II	Asthma	40	8	32 ∂ 2	91 ∂#2	29*	-	-
III	Asthma before steroids	16	5	36 ∂ 3	85 ∂#3	-	-	-
IV	Asthma with NS	19	3	38 ∂ 3	93 ∂#2	16	-	-
IV	Asthma without NS	21	5	28 ∂ 2	89 ∂#2	13*	-	-
V	Asthmatic symptoms	23	5	36 ∂#3	92 ∂#2	5	-	-

NS, nocturnal symptoms; *, metacholine challenge was not carried out in three subjects due to severe airway obstruction.

were assessed among the 40 asthmatic patients by asking whether wheezing, cough or chest tightness had disturbed the patient's sleep during the last week. Alveolar and bronchial NO output and serum levels of inflammatory markers were compared between patients with nocturnal symptoms and those without.

4.1.4 Alveolar and bronchial NO output in patients with asthmatic symptoms but normal lung function (V)

While recruiting the asthmatic patients, diagnostic evaluation of asthma was also conducted in 23 patients who had typical symptoms of asthma but who did not fulfil the lung function criteria for asthma. No other diseases causing their symptoms were found in routine clinical investigations. Alveolar and bronchial NO output, lung function, bronchial responsiveness, atopy and inflammatory markers in blood and urine were assessed in these subjects and compared to those of the 40 asthmatics and 40 healthy controls. Also correlations of alveolar and bronchial NO output to spirometric parameters and inflammatory markers were studied.

All the studies were approved by the Ethics Committee of Tampere University Hospital, and the subjects gave written informed consent. All subjects were non-smokers. Subject characteristics of patients in studies II – V are summarised in Table 1.

4.2 Methods

4.2.1 Exhaled NO measurement

Real-time measurements of exhaled NO concentration were made with a Sievers NOA 280 chemiluminescence NO analyser, with a sensitivity of < 1ppb, measurement range from < 1ppb to 500 000 ppb, response time of 200 ms and repeatability of ∂ 1 ppb. The lag time of the measuring and sampling system was 1.5 s with a sample gas flow of 200 ml/min. The test subjects were asked to breathe through a T-shaped two-way non-rebreathing valve with separate inhalation port, exhalation port and mouth port tube (Figure 12). The mouth port tube had fittings for the sample line to the NO analyser and for a line to a pressure transducer within the analyser. The exhalation port was equipped with a Luer adapter for installation of different mechanical flow restrictors. The inhalation port allowed inhalation of atmospheric air. The NO analyser was connected to a computer, which functioned as a feedback display unit to provide real-time visual guidance for the test subject to maintain exhalation pressure steady and at the desired level during the measurement. The computer also displayed mouth pressure and NO concentration curves against a time scale for final assessment of exhaled NO level. Different mechanical flow restrictors were used in the exhalation port of the breathing valve to obtain the desired exhalation flow rates at certain exhalation pressures. The flow characteristics of the restrictors were measured with an electric manometer and a mass flow meter at the Tampere Laboratory of Technical Research Centre of Finland.

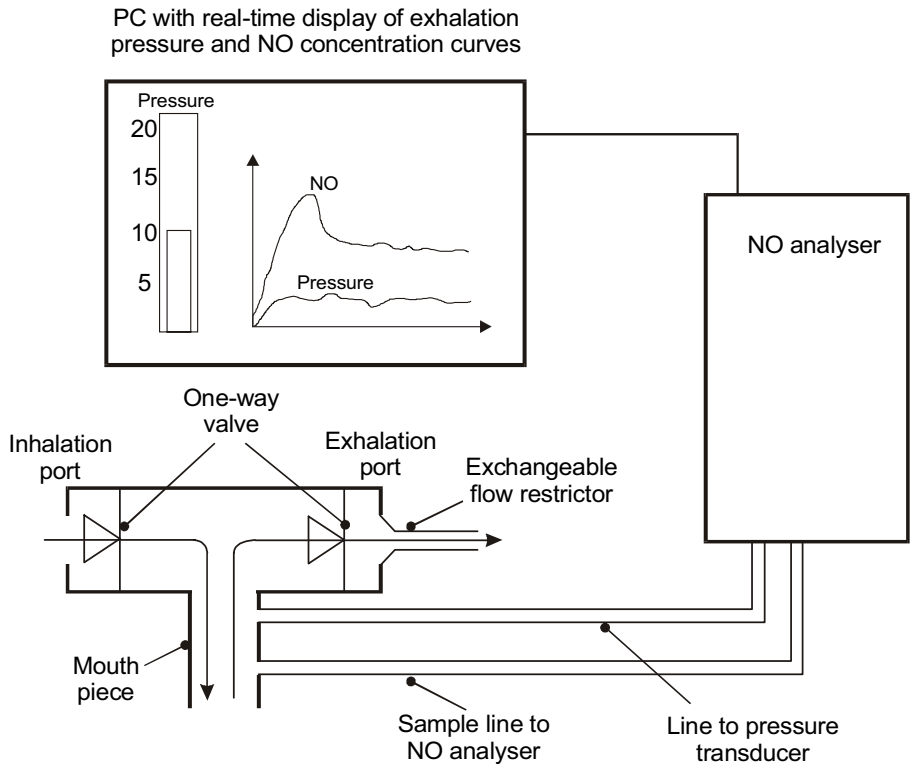

Figure 12. Schematic illustration representing the connections between breathing valve, NO analyser and PC as used in the study.

The analyser was calibrated daily using NO at known concentration (96.9, 101.0 or 103.0 ppm, AGA, Lidingö, Sweden) and the zero calibration was run before every subject using filtered NO-free air (Sievers Instruments filter). All subjects were seated during the measurement and nose clamps were not worn. Subjects were asked to inhale through the mouth to total lung capacity and then to exhale into the mouthpiece as long as they could maintain the exhalation pressure at the desired level. The NO concentration at the end-expiratory plateau during the last quarter of the total exhalation time was used in results. The mean of three successful NO measurements at each exhalation flow rate was used in results.

In studies applying the two-compartment model, exhaled NO was measured at flow rates of 40, 100, 175 and 370 ml/s with a mouth pressure of 9 cmH_2O. For each subject, NO output at every flow rate was calculated by multiplying the exhaled NO concentration by the flow rate used. A regression line was set between NO output and exhalation flow rate, and the slope and the intercept were calculated. The slope and the intercept are approximates of alveolar NO concentration and bronchial NO flux, respectively (Tsoukias and George 1998) (see 2.5 Mathematical Modelling of Pulmonary NO Dynamics for a more detailed introduction to the method). All four flow rates were used in the first study. However, as NO output is not a linear

function of exhalation flow rate at lower flow rates, only the three highest flow rates were used for the linear fitting in studies II – V.

4.2.2 Lung function

Spirometry (Vmax 20C, SensorMedics, Yorba Linda, CA, USA) was applied before and after 400 σg of inhaled salbutamol (Ventoline, GlaxoWellcome, Evreux, France), and the results were compared to normal values in the Finnish population (Viljanen et al. 1982).

Bronchial responsiveness was studied by allowing the patients to inhale increasing doses of methacholine and measuring spirometry after each dose (Nieminen 1992). A provocative dose of methacholine causing a 20 % decrease in FEV_1 ($PD_{20}FEV_1$) of less than 2.6 mg was considered indicative of bronchial hyperresponsiveness. As a 20 % decrease in FEV_1 was not reached in every patient, the $PD_{20}FEV_1$ of methacholine could not be calculated in all patients. Thus, the logarithm of dose-response slope (DRS = percent decline in FEV_1 divided by the total dose of methacholine inhaled) was calculated for each subject and used in statistical analysis (O'Connor et al. 1987).

The pulmonary diffusing capacity of carbon monoxide (DL_{CO}) was measured by the single-breath technique (Vmax 22 or model 2200, SensorMedics) (American Thoracic Society 1995) and the results compared to normal values in the Finnish population (Viljanen et al. 1982).

To analyse diurnal PEF variation in the diagnostic evaluation of asthma, the patients were requested to record their morning and evening PEF values during 2 weeks. During the second week, they were asked to measure PEF values before and after inhaling 200 σg of salbutamol.

4.2.3 Allergy testing

In patients sent for diagnostic evaluation of asthma, atopy was tested for by skin-prick test. Eleven common animal and aero-allergens (cat, dog, house dust mite (*Dematophagoides pteronyssinus* and *D. farinae*), alder, birch, timothy, smooth meadow-grass, mugwort, aspergillus, cladosporium) were tested (ALK-Abelló, Hørsholm, Denmark). A weal diameter at least 3 mm was considered a positive result.

4.2.4 Inflammatory markers in blood and urine

Venous blood was collected into Vacutainer K_3 EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA) for analysis of blood eosinophil count (analysed in the laboratory at Tampere University Hospital), and into 4 ml Vacutainer Hemogaard SST tubes (Becton Dickinson) to obtain serum. The sample in the serum tubes was allowed to clot for 60 minutes at 22º C and then centrifuged at 1300g for 10 min to separate serum. Serum level of IgE was measured by immunoluminometry (analysed in the laboratory at Tampere University Hospital). Serum levels of ECP, EPX and MPO were measured by radio-immuno-assay (ECP-RIA, EPX-RIA and MPO-RIA, Pharmacia AB, Uppsala, Sweden). The serum level of IL-6 was measured by enzyme-linked-

immonosorbent-assay (PeliPair ELISA, CLB, Amsterdam, Netherlands).

Urinary concentrations of EPX and LTE_4 were measured (EPX-RIA, Pharmacia AB, Uppsala, Sweden; LTE_4-EIA, Cayman Chemical, Ann Arbor, MI, USA) and normalised to the urinary concentration of creatinine to assess the urinary excretion rate of these markers.

Detection limits and inter-assay coefficients of variation in immunological analysis were 2 σg/l and 4.2% for ECP-RIA, 3 σg/l and 5.4% for EPX-RIA, 8 σg/l and 6.2% for MPO-RIA, 7.8 ng/l and 8.1% for LTE_4-EIA, 0.6 ng/l and 4.1% for IL-6 ELISA.

4.2.5 Asthmatic symptoms

Asthmatic symptoms were assessed by means of a written questionnaire. Cough, chest tightness and wheezing during the last week were each scored as none (0), on one day (1), on more than one day (2) or every day (3). Nocturnal symptoms were assessed by asking whether the above-mentioned symptoms had disturbed the patient's sleep during the last week. Nocturnal symptoms were scored as no nocturnal symptoms (0), symptoms on one night (1), symptoms on more than one night (2), or symptoms every night (3). The maximum symptom score was thus 12. The questionnaire and its English translation are given in Appendix 1.

4.2.6 Statistics

In studies concerning the two-compartment model, after log-transforming DRS of methacholine and serum levels of IgE, all parameters were normally distributed (Kolmogorov-Smirnov test) and parametric methods were used in statistical analysis. ANOVA with post-test (Bonferroni, Games-Howell, or Least significant difference) or t-test, where appropriate, was used to study differences in parameters of interest between subject groups. Correlation between different parameters was studied by Pearson's r. Repeated measures ANOVA with Dunnet's post-test or paired t-test, as appropriate, was used to study the effect of drug treatment on different parameters. A p-value of less than 0.05 was considered statistically significant. The results are given as mean ∂ SEM (standard error of the mean). SPSS 9.01 (SPSS Inc., Chicago, IL, USA) and InStat 3.05 (GraphPad Software Inc., San Diego, CA, USA) softwares were used in analysis.

5. SUMMARY OF RESULTS

5.1 Flow Dependency of Exhaled NO Concentration

The exhalation flow rate was found to have a major effect on exhaled NO concentration. Exhaled NO concentration decreased exponentially with increasing exhalation flow rate in patients with asthma or alveolitis and in healthy subjects (I) (Figure 13).

Patients with asthma had higher exhaled NO concentrations than healthy subjects at all four exhalation flow rates (40, 100, 175 and 370 ml/s). Patients with alveolitis had significantly increased exhaled NO concentration only at the two highest flow rates. Exhaled NO concentration was higher in asthmatics than in patients with alveolitis, but the difference was not significant at the highest flow rate (Figure 14).

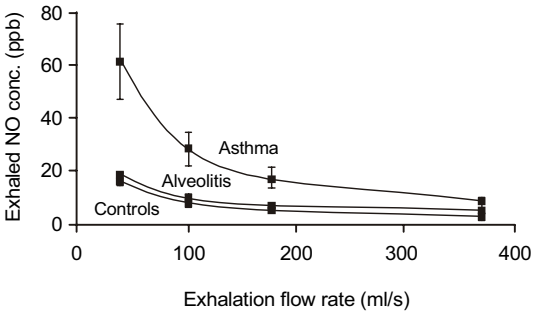

Figure 13. The exhaled NO concentration was highly dependent on the exhalation flow rate in healthy controls and patients with asthma or alveolitis. (Reprinted with permission from: Lehtimäki et al. 2000, Ann Med, 32: 417-423. ⊕ Taylor & Francis)

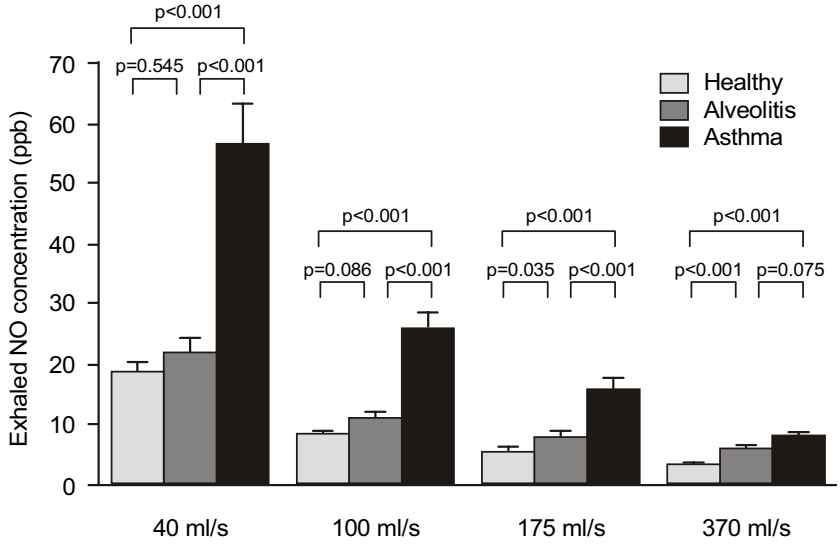

Figure 14. Exhaled NO concentration in 57 healthy controls, 17 patients with alveolitis and 40 patients with asthma at exhalation flow rates of 40, 100, 175 and 370 ml/s.

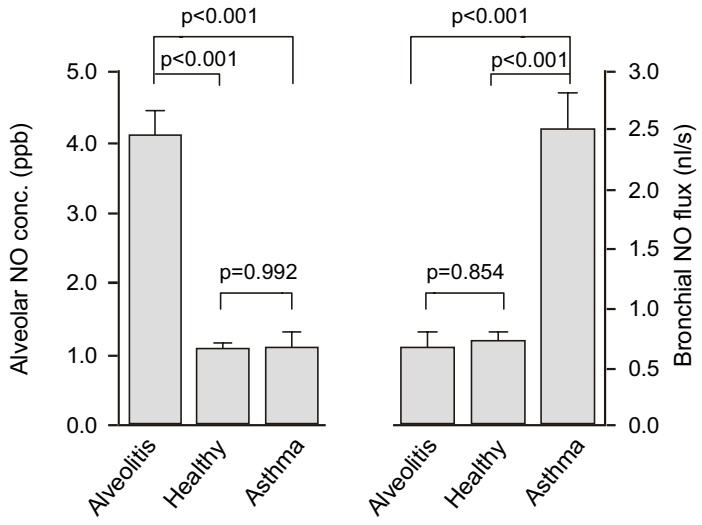

Figure 15. Alveolar NO concentration and bronchial NO flux in 17 patients with alveolitis, 40 patients with asthma and 57 healthy controls. (Reprinted with permission from: Lehtimäki et al. 2001, Am J Respir Crit Care Med 163: 1557-1561. ⊕ American Thoracic Society)

5.2 Alveolar and Bronchial NO Output in Asthma and Alveolitis

The exhaled NO concentration was measured at different exhalation flow rates, and the alveolar NO concentration and bronchial NO flux were calculated for each subject according to the mathematical model of pulmonary NO dynamics. Patients with asthma had increased bronchial NO flux but normal alveolar NO concentration, whereas those with alveolitis had increased alveolar NO concentration but normal bronchial NO flux, as compared with healthy controls (Figure 15) (I, II).

In patients with asthma, the increased bronchial NO flux correlated positively with bronchial hyperresponsiveness (Log DRS of methacholine), blood eosinophil count, serum levels of ECP and EPX, Log serum level of IgE and urinary excretion of EPX (Figure 16). The alveolar NO concentration correlated with none of these markers. Spirometric measures of lung function did not correlate to either bronchial or alveolar NO output (II, V).

In patients with alveolitis, the increased alveolar NO concentration correlated inversely with pulmonary diffusing capacity (DL_{CO} % of predicted), vital capacity and alveolar volume (V_A) (Figure 17). Bronchial NO flux did not correlate with these factors (II).

The increased alveolar NO concentration in patients with alveolitis may be attributed to one of two factors; either enhanced NO production in the alveolar tissue increases the diffusion rate of NO from alveolar tissue to alveolar air (increased $J_{NO,Alv}$), or the inflammatory / fibrosing process thickens the alveolar membrane and thereby lowers the diffusing capacity of NO from alveolar air to pulmonary capillaries (decreased $D_{NO,Alv}$). Judging from the reduced DL_{CO}

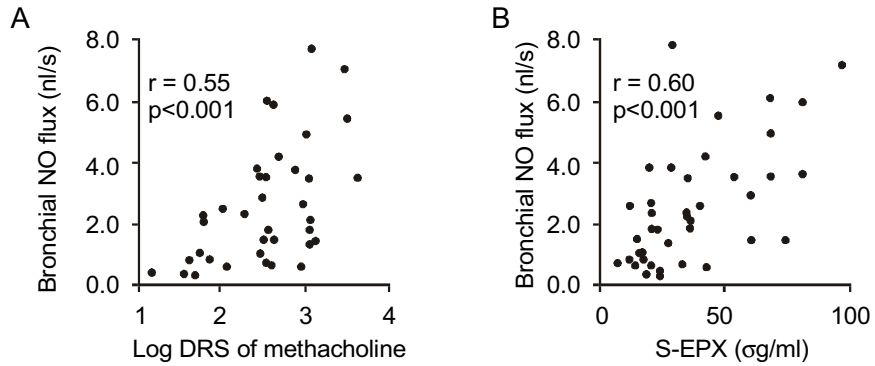

Figure 16. Correlation between bronchial NO flux and Log DRS of methacholine (A, n=37) and serum level of EPX (B, n=40) in patients with asthma. (Reprinted with permission from: Lehtimäki et al. 2001, Am J Respir Crit Care Med 163: 1557-1561. ⊕ American Thoracic Society)

values measured in these patients, $D_{NO,Alv}$ may also be assumed to be reduced. Whether enhanced $J_{NO,Alv}$ also participates in increasing the alveolar NO concentration in alveolitis was estimated using equation (6) (page 37) to calculate $J_{NO,Alv}$ based on C_{Alv} and DL_{CO} measurements. In healthy controls and patients with asthma DL_{CO} was not measured but was assumed to equal the age- and sex-dependent normal value, and $J_{NO,Alv}$ was also calculated in these subject groups. Patients with alveolitis had significantly higher $J_{NO,Alv}$ than those with asthma or healthy controls (II).

5.3 The Effect of Treatment on Alveolar and Bronchial NO Output in Asthma and Alveolitis

The increased bronchial NO flux in patients with asthma was reduced back to normal level by treatment with inhaled fluticasone. However, the treatment had no effect on alveolar NO concentration which was normal already before treatment. Bronchial

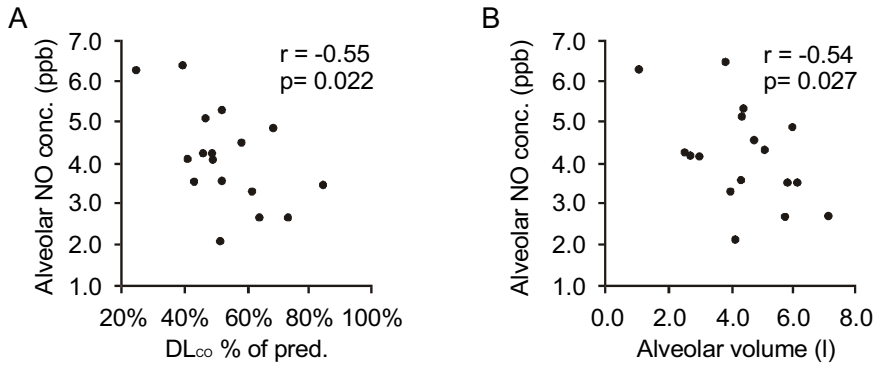

Figure 17. Correlation between alveolar NO concentration and DL_{CO} (A) and alveolar volume (B) in 17 patients with alveolitis. (Reprinted with permission from: Lehtimäki et al. 2001, Am J Respir Crit Care Med 163: 1557-1561. ⊕ American Thoracic Society)

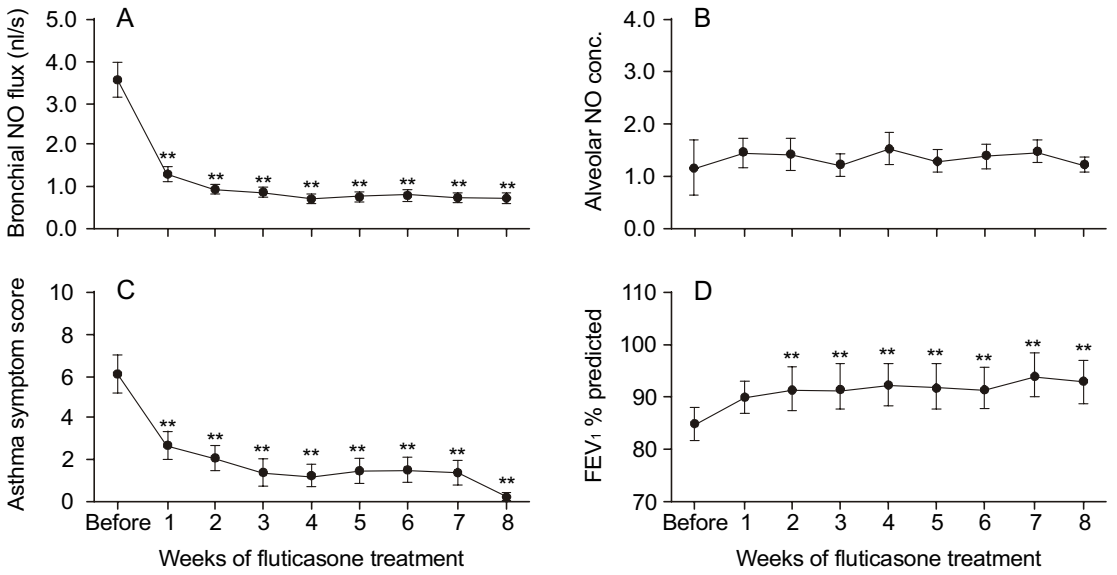

Figure 18. Bronchial NO flux (A), alveolar NO concentration (B), asthma symptom score (C) and FEV_1 % of predicted (D) before and during treatment with inhaled fluticasone in 16 patients with asthma. ** $p<0.01$ as compared with baseline. (Reprinted with permission from: Lehtimäki et al. 2001, Eur Respir J 18: 635-639. ⊕ ERS Journals Ltd)

NO flux was significantly decreased already after one week of treatment, and was no longer statistically different from that in healthy controls. Inhaled fluticasone also improved FEV_1 and lowered the asthma symptom score and serum levels of ECP and EPX in these subjects. Bronchial NO flux decreased most dramatically during the first 2 to 3 weeks of treatment, simultaneously with a decrease in symptom score, but the

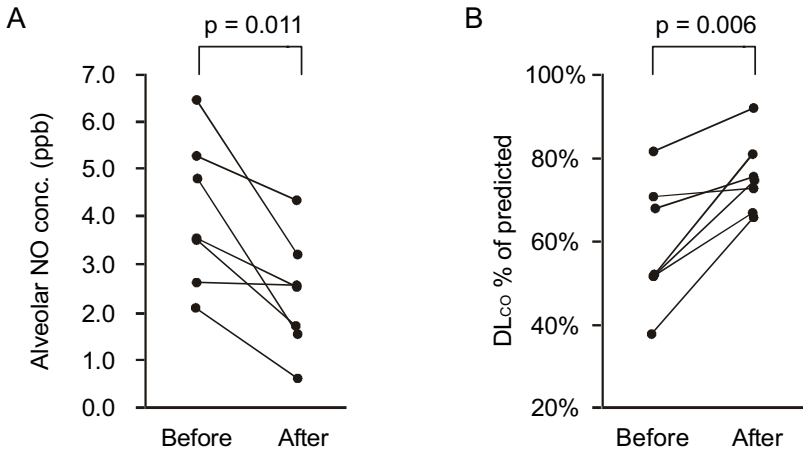

Figure 19. Alveolar NO concentration (A) and DL_{CO} % of predicted (B) in 7 patients with alveolitis before and after 2 months of allergen avoidance or drug treatment. (Reprinted with permission from: Lehtimäki et al. 2001, Am J Respir Crit Care Med 163: 1557-1561. ⊕ American Thoracic Society)

increase in FEV_1 was significant only after two weeks of treatment (Figure 18) (III).

Allergen avoidance and/or drug treatment for two months improved the pulmonary diffusing capacity and reduced the alveolar NO concentration in the 7 patients with alveolitis (Figure 19). After treatment, the alveolar NO concentration was still higher than in healthy controls, and the pulmonary diffusing capacity was lower than normal. There was no change in bronchial NO flux during the treatment (II).

5.4 Relation of Alveolar and Bronchial NO Output to Nocturnal Symptoms in Patients with Asthma

Of the 40 patients with asthma, 19 reported nocturnal symptoms and 21 had none. Patients with nocturnal symptoms had higher alveolar NO concentrations than those without nocturnal symptoms or healthy controls. However, alveolar NO concentration in asthmatic patients with nocturnal symptoms was still considerably lower than in patients with alveolitis. As compared with healthy controls, bronchial NO flux was increased in both asthma groups, and there was no difference between the asthma groups (Figure 20) (IV).

Serum levels of EPX were higher in both asthma groups than in healthy controls. The levels were somewhat higher in asthmatics with nocturnal symptoms than in those without nocturnal symptoms, but the difference was not statistically significant. As compared with controls, serum levels of ECP were increased only in patients with nocturnal symptoms, not in patients without nocturnal symptoms. Serum levels of MPO were higher in patients with nocturnal symptoms than in those without, but neither of the asthma groups differed significantly from controls. Serum levels of IL-6 were higher in patients with nocturnal symptoms than in those without nocturnal symptoms or healthy controls (IV).

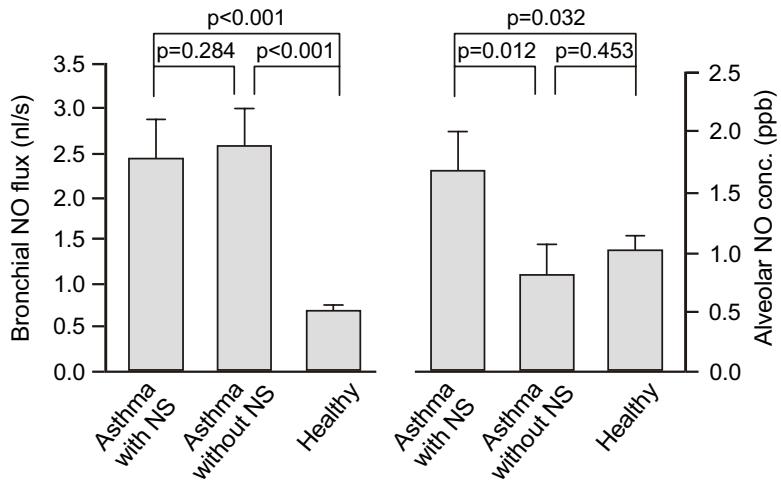

Figure 20. Bronchial NO flux and alveolar NO concentration in 19 asthmatics with nocturnal symptoms (NS), 21 asthmatics without nocturnal symptoms and 40 healthy controls. (Reprinted with permission from: Lehtimäki et al. 2002, Eur Respir J 20: 841-845. ⊕ ERS Journals Ltd)

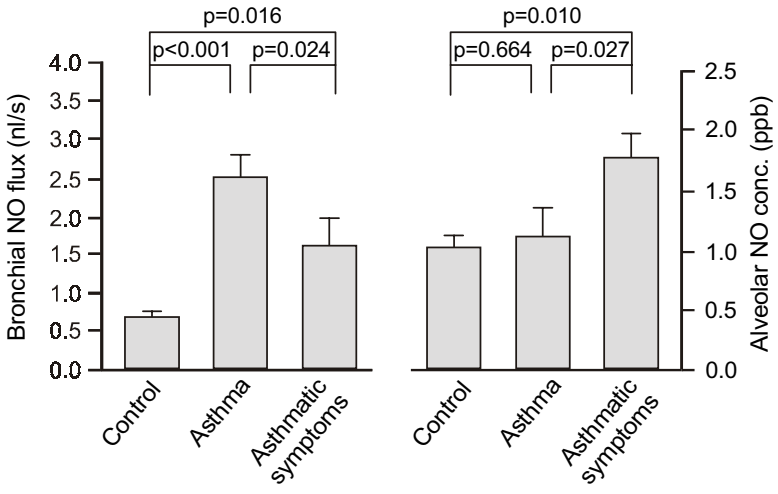

Figure 21. Bronchial NO flux and alveolar NO concentration in 40 healthy controls, 40 patients with asthma and 23 patients with asthmatic symptoms but normal lung function.

5.5 Alveolar and Bronchial NO Output in Patients with Asthmatic Symptoms but Normal Lung Function

Of the 65 patients with asthmatic symptoms recruited into the study, 40 met the diagnostic lung function criteria for asthma. Twenty-three had asthmatic symptoms but did not fulfil the lung function criteria and no other underlying diseases were found. In the patients with asthmatic symptoms but normal lung function, the alveolar NO concentration was higher than in healthy controls or in patients with asthma. However, the alveolar NO concentration in these patients was still considerably lower than in patients with alveolitis. Also bronchial NO flux was increased in these subjects, but was lower than in patients with asthma (Figure 21) (V).

In patients with asthmatic symptoms but normal lung function, alveolar NO concentration correlated positively with

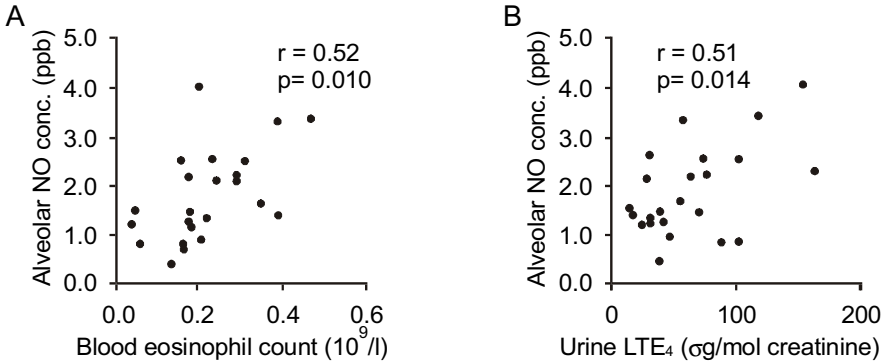

Figure 22. Correlation between alveolar NO concentration and blood eosinophil count (A) and urinary excretion of LTE_4 (B) in 23 patients with asthmatic symptoms but normal lung function.

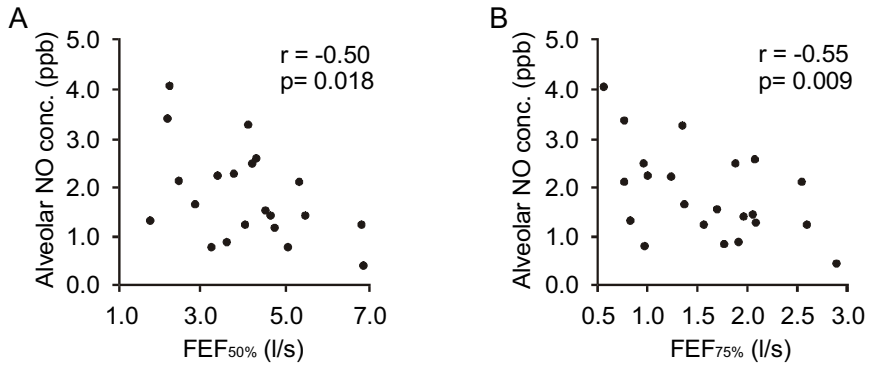

Figure 23. Correlation between alveolar NO concentration and $FEF_{50\%}$ (A) and $FEF_{75\%}$ (B) in 23 patients with asthmatic symptoms but normal lung function.

blood eosinophil count and urinary LTE_4 (Figure 22). Alveolar NO concentration correlated negatively with measures of small-airway function ($FEF_{50\%}$ and $FEF_{75\%}$) (Figure 23). Bronchial NO flux correlated positively with bronchial hyperresponsiveness (Log DRS of methacholine) (V).

6. DISCUSSION

Theoretical considerations regarding the two-compartment model of pulmonary NO dynamics suggest that the alveolar and bronchial contributions to exhaled NO can be calculated by measuring the exhaled NO concentration at multiple exhalation flow rates. Studies I and II of this series were the first publications applying the model to healthy controls and patients with alveolar inflammation (alveolitis) or bronchial inflammation (asthma) in clinical setting. The main finding was that assessment of alveolar and bronchial NO output by the multiple flow rate method differentiates between bronchial and alveolar / peripheral components in pulmonary inflammation.

6.1 Flow Rate Dependency of Exhaled NO Concentration

In the present study, exhaled NO concentration was found to be inversely related to exhalation flow rate in both healthy and diseased subjects. The results are in line with previous reports (Högman et al. 1997, Silkoff et al. 1997) and confirm the role of conducting airways as a major source of exhaled NO.

Exhaled NO concentration is a sum of both alveolar and bronchial NO output. At higher exhalation flow rates, the exhaled NO concentration reflects predominantly the alveolar NO concentration, as exhaled air travels through the conducting airways very rapidly and there is little time for more NO to be taken up from the bronchial mucosa. At lower exhalation flow rates, the passage of exhaled air through the conducting airways takes longer and there is more time for NO to diffuse from the bronchial mucosa to the luminal air. At low exhalation flow rates, the exhaled NO concentration thus reflects predominantly bronchial NO dynamics.

In view of the above said, the magnitude of the chosen exhalation flow rate is clearly a critical factor determining where in the respiratory tract inflammatory changes can be detected. If a high flow rate is chosen, the exhaled NO concentration reflects NO dynamics mainly in the alveolar region and is not sensitive to detect increased bronchial NO production. Conversely, if a low flow rate is chosen, the exhaled NO concentration will reflect mainly the bronchial compartment and will be insensitive to changes in alveolar NO dynamics. The exhaled NO concentration at the currently recommended flow rate of 50 ml/s reflects mostly bronchial NO output and cannot be used to study alveolar NO dynamics. This conception is supported by the results of this series. Exhaled NO concentration at flow rates of 40 and 100 ml/s could differentiate asthmatics from healthy controls and from patients with alveolitis, but patients with alveolitis could not be distinguished from healthy subjects. At higher flow rates of 175 and 370 ml/s also patients with alveolitis could be differentiated from healthy subjects, but the difference between asthma and alveolitis was no longer significant at the highest flow rate. It is therefore likely that alveolar inflammation is missed by measuring exhaled NO concentration only at

a flow rate of 50 ml/s, but at higher flow rates alveolar inflammation cannot be differentiated from bronchial inflammation. It is here that the multiple flow rate method shows its superiority, as it can be used to assess the alveolar and bronchial components in pulmonary inflammation separately.

6.2 Critique of the Two-Compartment Model

The two-compartment model of pulmonary NO dynamics was developed to allow more precise interpretation of exhaled NO measurements. Earlier models regarded only the alveolar region as a source of NO and neglected the bronchial tree (Hyde et al. 1997). However, experimental observations showed an inverse relation between exhalation flow rate and exhaled NO concentration, suggesting that the conducting airways are a major contributor to the exhaled NO concentration (Högman et al. 1997, Silkoff et al. 1997). The two-compartment model considers both alveolar and bronchial regions as possible sources of NO, and also provides a theoretical means to separate between alveolar and bronchial NO production (Tsoukias and George 1998).

To retain mathematical simplicity, the model is based on a robust simplification of the human lung and cannot therefore be considered to describe pulmonary NO dynamics in all its details. The millions of tiny alveoli and alveolar sacs together with respiratory bronchioles are reduced to a single huge alveolus, and the branching bronchial tree is represented by a single rigid cylindrical tube (Tsoukias and George 1998). Alveolar and bronchial NO parameters are therefore only average values describing the whole alveolar or bronchial compartment, respectively, and local differences in NO dynamics within a compartment cannot be assessed.

As all the alveoli are represented by a single well-mixed alveolus, the model neglects possible differences in the NO dynamics of parallel alveoli and also the sequential filling of the alveoli. During normal respiration, the alveoli which are filled first during inhalation are emptied last during exhalation (usually the apical region of the lung), while the alveoli which are filled last are emptied first (usually the basal region) (Fukuchi et al. 1980, Tsoukias et al. 2000b). Thus, air from basal alveoli predominates in the early expiration, whereas air from apical alveoli predominates in the late phase. If there is a significant difference in alveolar NO concentration between basal and apical lung, this might cause a downward or upward slope in the plateau NO concentration during a single exhalation. A model taking into account the sequential filling of the lung might be able to differentiate between apical and basal inflammatory processes.

The two-compartment model considers convection as the only mode of axial gas transport and neglects axial diffusion of NO in the gas phase (e.g. diffusion of NO from bronchial air with higher NO concentration backwards to alveolar air with lower NO concentration). However, diffusion has been shown to be a major contributor to gas transport particularly in the very small airways and the alveolar region. It has been suggested that neglect of axial diffusion in the two-compartment model leads to a four-

fold underestimation of maximal bronchial NO flux (Shin and George 2002a). However, this underestimation depends markedly on the significant contribution of small airways to bronchial NO flux. If bronchial NO flux is mainly derived from the large airways, as in healthy subjects (Silkoff et al. 1998), neglecting axial diffusion will cause no major underestimation of bronchial NO flux (Shin and George 2002a).

The two-compartment model assumes alveolar and bronchial NO parameters to be constant while the lung volume decreases during exhalation. There are, however, data showing that the alveolar diffusing capacity of NO ($D_{NO,Alv}$) decreases with decreasing alveolar volume during exhalation (Borland and Higenbottam 1989, Tsoukias et al. 2000a). According to equation (1) (page 33), the alveolar NO concentration is inversely related to $D_{NO,Alv}$. The alveolar NO concentration should therefore increase during a single exhalation as the decreasing alveolar volume also reduces $D_{NO,Alv}$. This would tend to increase the total exhaled NO concentration towards end-exhalation. However, during the plateau phase of the exhaled NO curve, the NO value usually decreases slightly with decreasing pulmonary volume towards end-exhalation (Silkoff et al. 1997, Tsoukias et al. 1998). It is therefore suggested that the flux of NO from alveolar tissue to alveolar air ($J_{NO,Alv}$) would also decrease with decreasing alveolar volume such that the ratio between $J_{NO,Alv}$ and $D_{NO,Alv}$ would be constant during exhalation (Tsoukias and George 2001a). This would lead to a constant alveolar NO concentration, as C_{Alv} is equal to $J_{NO,Alv}$ / $D_{NO,Alv}$ (see equation (1) on page 33).

Although the bronchial compartment in the two-compartment model is a rigid tube whose volume does not change during the respiratory cycle, the small bronchioles very likely decrease in diameter as pulmonary volume is reduced during exhalation. The decreased diameter of the small bronchioles would also reduce the mucosal surface area of the bronchial compartment. Bronchial NO flux is determined by the bronchial wall NO concentration and the bronchial diffusing capacity of NO, which in turn is dependent on the mucosal surface area available for diffusion (Tsoukias and George 1998). The decreasing bronchial surface area during exhalation would therefore tend to reduce the bronchial NO flux and exhaled NO concentration towards end-exhalation (Tsoukias and George 2001a). This might be the explanation for the observed decrease in exhaled NO concentration during the plateau phase of the NO curve of a single exhalation. The exhaled NO concentration has been found to decrease after induction of bronchoconstriction (de Gouw et al. 1998, Scollo et al. 2000, Terada et al. 2001), while attenuation of the bronchoconstriction with inhaled η_2-agonists increases exhaled NO levels back to normal (Ho et al. 2000b). These changes in exhaled NO concentration can also be explained by corresponding changes in the bronchial mucosal surface area after bronchoconstriction or bronchodilatation.

While it is evident that this simplified model cannot describe pulmonary NO dynamics in all detail, the important question is whether it still yields clinically useful information. For example, the widely used measures of pulmonary diffusing capacity are also based on a robustly

simplified model of the lung, but the clinical value of these measurements is indisputable. The results of the present study suggest that the relatively simple equations of the two-compartment model do indeed provide clinically useful information and allow differentiation between alveolar and bronchial inflammation. Further studies are needed to establish whether taking into account the parameters neglected by the two-compartment model would provide additional clinically useful information on pulmonary NO dynamics.

6.3 Exhaled NO Measurement at Multiple Flow Rates in Assessing Alveolar and Bronchial Inflammation

The exhaled NO concentration was measured at multiple exhalation flow rates in patients with asthma and alveolitis. Using the linear equation derived from the two-compartment model (Eq (5) on page 36), alveolar NO concentration and bronchial NO flux were calculated for each subject. The increased alveolar NO concentration in alveolitis and the increased bronchial NO flux in asthma suggest that exhaled NO measurement at multiple exhalation flow rates can be used to differentiate between alveolar and bronchial inflammation. Further, alveolar NO concentration and bronchial NO flux correlated with disease severity and responded to anti-inflammatory treatment in alveolitis and asthma. However, biopsy studies are needed to further assess the relation of alveolar and bronchial NO parameters to NOS expression and indices of inflammatory activity in the alveolar and bronchial compartments.

6.3.1 Alveolar and bronchial NO in asthma

Asthmatic patients had approximately 3.5 times higher bronchial NO flux than healthy subjects, but there was some overlap in individual values between the groups. The mean alveolar NO concentration in patients with asthma was not different from that in healthy subjects, but variation in asthmatics was somewhat higher.

An increased bronchial NO flux in asthma has lately also been found by other study groups utilising the multiple flow rate method (Silkoff et al. 2000, Högman et al. 2001, Delclaux et al. 2002). If only relatively high exhalation flow rates (> 50 ml/s) are used, exhaled NO can be partitioned into alveolar NO concentration and bronchial NO flux by the linear equation (Eq (5) on page 36) (Tsoukias and George 1998). However, including also very low flow rates (down to 5 - 10 ml/s) allows application of the non-linear equation (Eq (2), page 35) to divide bronchial NO flux into its two components: bronchial wall NO concentration and bronchial diffusing capacity of NO (Högman et al. 2000, Silkoff et al. 2000). As only relatively high exhalation flow rates were used in the present study, it was not possible to address the question whether the increased bronchial NO flux in asthma was due to increased airway wall NO concentration or increased bronchial diffusing capacity of NO. By also including very low flow rates, Silkoff and colleagues found an increased bronchial wall

diffusing capacity of NO and a slightly but not significantly increased bronchial wall NO concentration in asthma (Silkoff et al. 2000). Högman and colleagues reported that the increased bronchial NO output in asthma is due to an increase in both bronchial wall NO concentration and the bronchial wall diffusing capacity of NO (Högman et al. 2002b).

Increased bronchial wall NO concentration in asthma can be argued to represent a higher NO production rate caused by enhanced iNOS expression in the asthmatic airways (Hamid et al. 1993, Saleh et al. 1998). The increased bronchial wall diffusing capacity of NO is more difficult to interpret in terms of pathological changes in asthmatic airways. In theory, the bronchial wall diffusing capacity of NO can be increased due to either physical changes in the mucosa improving the actual diffusivity of NO, or an increase in the mucosal surface area producing NO (Tsoukias and George 1998, Silkoff et al. 2000). Silkoff and colleagues proposed that the main source of bronchial NO in healthy subjects would be the proximal largest airways; lobar bronchi, main bronchi and trachea (Silkoff et al. 1998, Silkoff et al. 2000). They attribute the increased bronchial wall diffusing capacity of NO in asthma to an increased total airway area producing NO, i.e. spreading of the NO-producing area also to smaller airways. This increased NO production area would be a reflection of NO-related bronchodilatory defence mechanism against bronchoconstriction. So far there are no means of separating between production area and physical diffusivity as components in the bronchial wall diffusing capacity of NO. More studies with advanced measurement techniques are needed to establish how these two factors are affected in asthmatic airway inflammation.

In this study bronchial NO flux correlated positively with several markers of asthmatic inflammation, for example blood eosinophil count, serum levels of ECP and EPX, and urinary excretion of EPX. This is in line with results obtained by Högman and colleagues showing a positive correlation between bronchial NO flux and percentage of sputum eosinophils in asthma (Högman et al. 2001). In addition, several studies using the single flow rate method have found exhaled NO to correlate with direct indices of eosinophilic inflammation in sputum, BAL and bronchial biopsies (Jatakanon et al. 1998, Chan-Yeung et al. 1999, Lim et al. 1999, Mattes et al. 1999, Payne et al. 2001, van den Toorn et al. 2001, Warke et al. 2002). These results support the role of bronchial NO flux as a measure of bronchial inflammation in asthma. There was also a positive correlation between bronchial NO flux and serum level of IgE in this study. No previous studies have assessed the relation between these measures, but studies using the single flow rate method have shown a correlation between exhaled NO concentration and the serum level of IgE (Simpson et al. 1999). The positive correlation between bronchial NO flux and serum IgE levels is in line with studies showing higher exhaled NO levels in atopic than non-atopic asthmatics.

Bronchial NO flux correlated positively with bronchial hyperresponsiveness (Log DRS of methacholine). Thus, the bronchial NO flux was higher in patients with more responsive airways. The exhaled NO concentration at a single flow rate has also

been found to correlate with bronchial responsiveness (Dupont et al. 1998, Lim et al. 1999). Asthmatic inflammation induces bronchial iNOS expression and also enhances bronchial responsiveness. The correlation between bronchial NO flux and hyperresponsiveness is thus what would be expected. However, Silkoff and colleagues showed a positive correlation between PC_{20} of methacholine and $D_{NO,Br}\Delta C_W$ (−bronchial NO flux) after treatment with inhaled glucocorticoids, i.e. bronchial NO flux after steroid treatment was higher in patients with less reactive airways. As inhaled glucocorticoids lower iNOS but not cNOS expression in the airways (Saleh et al. 1998), the authors concluded that the higher bronchial NO flux after steroids would reflect more intense bronchoprotective nNOS expression. This would explain the less reactive airways in subjects with higher bronchial NO flux after steroid treatment.

The increased bronchial NO flux but normal average alveolar NO concentration in asthma found in this study is in line with the classic view of asthma as an inflammatory disease of the large airways. Other study groups have likewise reported that alveolar NO concentration in asthmatics does not differ from that in healthy controls (Högman et al. 2001, Delclaux et al. 2002). However, alveolar inflammation has been found in transbronchial biopsies from patients with stable chronic asthma (Kraft et al. 1996) and nocturnal asthma is associated with further enhancement of alveolar inflammation (Martin et al. 1991). This is in line with the increased alveolar NO concentration seen in asthmatics with nocturnal symptoms in the present study. As bronchial NO flux in asthmatics with nocturnal symptoms was not higher than in patients without these symptoms, exhaled NO measurement at a single flow rate could not differentiate between these two asthma groups. The results suggest that measurement of alveolar NO concentration could be used to assess the peripheral component in asthmatic inflammation associated with nocturnal asthma.

6.3.2 Alveolar and bronchial NO in alveolitis

Patients with CFA or EAA had increased alveolar NO concentrations but normal bronchial NO flux. Girgis and colleagues have reported increased alveolar NO concentration but slightly decreased bronchial NO flux in patients with FASSc (Girgis et al. 2002). The increased alveolar NO concentration in patients with alveolitis in the present study was not entirely explained by the decreased pulmonary diffusing capacity of NO, but the results suggest that the actual alveolar production rate of NO was also increased. The increased production rate of NO is consistent with the iNOS expression found in the alveolar epithelium of patients with CFA (Saleh et al. 1997).

The alveolar NO concentration in alveolitis correlated negatively with DL_{CO} and alveolar volume. Also Girgis and colleagues found a negative correlation between alveolar NO concentration and DL_{CO} in patients with FASSc (Girgis et al. 2002). The correlation between alveolar NO concentration and DL_{CO} might be explained by a thickening of the alveolar membrane reducing diffusion of both CO and NO. A

reduced diffusing capacity of NO would increase the alveolar NO concentration (see Eq (1) on page 33), and therefore decreased DL_{CO} and increased alveolar NO concentration would both reflect the same pathophysiological feature, namely impaired diffusion of gases in the alveolar region. However, the results of the present study suggest that the alveolar production rate of NO was also increased. This is supported by the increased iNOS expression in the alveolar epithelium of patients with CFA (Saleh et al. 1997). Thus, increased alveolar NO concentration might not only reflect a decreased alveolar diffusing capacity of NO but would also serve as a marker of inflammatory activity in the alveolar compartment. There are also animal data suggesting that an increased alveolar production rate of NO might be one of the factors causing inflammation and excess fibrosis in alveolar tissue (de Rezende et al. 2000, Giri et al. 2000), and might thus actually be one of the factors causing decreased DL_{CO} in fibrosing alveolitis.

In the present work, the alveolar NO concentration was not compared to direct measures of pulmonary inflammation in patients with alveolitis. Previously, the exhaled NO concentration at a single flow rate has been compared to inflammatory indices in BAL fluid in patients with FASSc. The exhaled NO concentration was higher in patients with signs of active inflammation in BAL than in patients with normal BAL, and there was a correlation between exhaled NO concentration and BAL lymphocyte cell count (Paredi et al. 1999). In CFA or FASSc, the iNOS expression in alveolar tissue is more pronounced in patients with an active inflammatory pattern in biopsies as compared with patients having end-stage fibrosis (Saleh et al. 1997, Romanska et al. 2000b). An increased alveolar NO concentration might thus be used as a marker of on-going active inflammation in patients with fibrosing alveolitis. As inflammatory-stage rather than end-stage fibrosis is thought to be more responsive to drug treatment (American Thoracic Society and European Respiratory Society 2000), measurement of the alveolar NO concentration might serve as a useful tool in identifying patients most likely to benefit from drug treatment.

Inflammation in EAA is not restricted to alveolar tissue only; also bronchiolitis and peribronchiolar granulomas are typically found in the disease. However, bronchial NO flux was not increased in these subjects. There are two possible reasons for this. Firstly, the most peripheral bronchioles are included in the alveolar compartment in the two-compartment model of pulmonary NO dynamics. Secondly, the exhaled NO concentration reflects mostly NO production in the airway epithelium rather than in deeper tissue layers (Gabbay et al. 1999). NO produced in the peribronchiolar granulomas might thus not, due to the relatively long distance, diffuse into the airway lumen in high enough quantities to affect bronchial NO flux. Studies with larger numbers of subjects and assessment of bronchiolitis are needed to establish whether profound bronchiolitis increases bronchial NO flux in EAA.

6.3.3 Responsiveness of alveolar and bronchial NO output to alleviation of inflammation

There is a clinical need for a marker of airway inflammation which would allow assessment of the efficacy of anti-inflammatory treatment in lung diseases. In asthma, the exhaled NO concentration measured at a single exhalation flow rate decreases dose-dependently during treatment with inhaled glucocorticoids, while discontinuation of the treatment increases the exhaled NO level together with increase in symptoms (Kharitonov et al. 1996b, Silkoff et al. 2001, Jones et al. 2002, Kharitonov et al. 2002). Patients with CFA using anti-inflammatory medication show lower levels of exhaled NO than patients without such medication (Paredi et al. 1999). These observations suggest that NO production might serve as a surrogate marker for inflammatory activity during drug treatment.

It was found in the present study that anti-inflammatory treatment reduces bronchial NO flux in asthma and the alveolar NO concentration in alveolitis. The responsiveness of these parameters to anti-inflammatory treatment in the compartment where the disease process is considered mainly to take place supports the role of alveolar and bronchial NO parameters as inflammatory markers of the respective compartments.

In asthma, inhaled fluticasone reduced bronchial NO flux but had no effect on alveolar NO concentration. This is understandable, as the alveolar NO concentration was already normal before treatment. Silkoff and colleagues have reported that the decreased bronchial NO flux in asthma following inhaled glucocorticoids is due to a decreased bronchial wall NO concentration (Silkoff et al. 2000). This is in line with biopsy studies showing decreased iNOS expression in asthmatic airways after inhaled glucocorticoids (Saleh et al. 1998). Glucocorticoids may reduce iNOS expression in at least two ways; directly by interfering with the transcription and translation of iNOS (inhibition of NF-ρB or destabilisation of iNOS mRNA) (Kleinert et al. 1996, Korhonen et al. 2002), or indirectly by lowering the production rate of inflammatory cytokines needed to induce iNOS expression in airway cells.

The effect of inhaled fluticasone was rapid. A significant decrease in bronchial NO flux was already evident at the first control visit after one week of treatment, and bronchial NO flux was no longer statistically different from that in healthy controls. Other studies have shown that a high dose of inhaled budesonide reduces the exhaled NO concentration already after 6 hours (Tsai et al. 2001). In the present study, bronchial NO flux decreased simultaneously with asthmatic symptoms and preceded the increase in FEV_1.

In patients with alveolitis, anti-inflammatory treatment reduced the alveolar NO concentration but had no effect on bronchial NO flux. There was also an increase in pulmonary diffusing capacity during the treatment. However, even after the treatment, alveolar NO concentration was higher than in healthy controls, but the diffusing capacity in patients was still lower than normal. Anti-inflammatory treatment may reduce the alveolar NO concentration

by two different mechanisms. It might lower the actual alveolar production rate of NO, or reduce alveolar oedema and therefore increase the diffusion rate of NO from alveolar air to pulmonary blood to be scavenged by haemoglobin.

These results suggest that bronchial NO flux and alveolar NO concentration could be used in assessing the efficacy of anti-inflammatory treatment in patients with bronchial or alveolar inflammation.

6.3.4 Alveolar and bronchial inflammation in patients with asthmatic symptoms but normal lung function

In patients with asthmatic symptoms but normal lung function, bronchial NO flux was higher than in healthy subjects but lower than in patients with asthma. This suggests that the asthmatic symptoms group had bronchial inflammation but that this was milder than in asthmatic subjects. This is in line with other studies in such patients, showing sputum eosinophils to be increased but lower than in asthma (Gibson et al. 1989, Rytilä et al. 2000). Bronchial responsiveness was also increased in the asthmatic symptoms group but was lower than in patients with asthma. The exhaled NO concentration at a single exhalation flow has been shown to be increased in a similar group of patients with asthmatic symptoms but normal lung function (Ekroos et al. 2002). These results suggest that functional criteria of asthma are not sufficient to detect all the patients with airway inflammation, but inflammatory markers are needed.

In addition, the alveolar NO concentration was slightly increased in the asthmatic symptoms group, and it correlated negatively with the spirometric indices of small airway function. This would imply that the asthmatic symptoms group had a more peripheral inflammation than patients fulfilling the current diagnostic criteria of asthma. The results also suggest that among patients with asthma-like symptoms, those with more central airway inflammation show impaired large airway function (changes in PEF and FEV_1) and fulfil the lung function criteria for asthma, while subjects with more peripheral inflammation and milder inflammation in the large airways do not have impaired large airway function and thus do not fulfil the current criteria for asthma. However, further studies with biopsies would help to validate alveolar and bronchial NO output measures against inflammatory activity in these patient groups.

6.3.5 Critique of the Present Method

In the present study, alveolar NO concentration and bronchial NO flux were calculated using the linear equation (Eq (5) on page 36) of the two-compartment model based on exhaled NO concentrations measured at exhalation flow rates of 100, 175 and 370 ml/s (also 40 ml/s in the first paper). The exhalation flow rates were achieved by means of different mechanical flow restrictors for each flow rate at the exhalation port of the breathing valve, and the patients were requested to keep exhalation pressure steady at a target level. The achieved exhalation flow rate is

dependent on the patient's ability to maintain exhalation pressure steady at the desired level. There was thus probably some deviation in the flow rate values achieved, which might have affected the results.

The alveolar NO concentrations in healthy subjects and asthmatics in the present study are somewhat lower than those published by other study groups using the linear fitting method (Tsoukias et al. 1998, Högman et al. 2001, Delclaux et al. 2002, Girgis et al. 2002). Differences in measurement technique (e.g. the exhalation flow rates used) might be the reason. The linear function between NO output and exhalation flow rate is based on the assumption that the NO concentration in the bronchial luminal air is not significantly higher than the alveolar NO concentration, so that the bronchial NO flux would equal its maximum value ($(C_W-C_{Alv}) \Delta D_{NO,Br}$). At very high exhalation flow rates this is roughly the case, but at lower flow rates the NO concentration in the bronchial luminal air increases and diffusion of NO from bronchial mucosa to luminal air decreases. Thus, the linear fitting between NO output and exhalation flow rate should be used only at such high flow rates that the assumption of maximum bronchial NO flux is justified and the relation between NO output and exhalation flow rate is linear. How high such flow rates are, depends on the magnitude of bronchial NO flux. The higher the bronchial NO flux, the higher should be the flow rates. Usually only flow rates higher than 50 ml/s are recommended for the linear fitting. Based on the present data, 100 ml/s might be a useful lower limit especially in asthmatics.

Approximates of alveolar NO concentration and bronchial NO flux calculated by the linear fitting method are affected by the magnitude of the chosen exhalation flow rates. Inclusion of too low flow rates causes overestimation of the alveolar NO concentration and underestimation of the bronchial NO flux. Exclusion of the lowest exhalation flow rate (40 ml/s) in the first paper of the present series would increase the bronchial NO flux from 2.6 to 2.8 nl/s and lower the alveolar NO concentration from 1.5 to 0.7 ppb in patients with asthma. This might also be explained in part by inaccuracies in exhalation flow rates.

At higher flow rates the problem of overestimating the alveolar NO concentration and underestimating the bronchial NO flux diminishes. However, at higher flow rates the duration of a single exhalation is shorter by reason of the limited pulmonary capacity, and also exhaled NO concentrations are lower. The shorter duration of exhalation and low NO levels cause inaccuracy in NO measurement due to the limited performance of current NO analysers. Differences in chosen flow rates might cause differences in results between study groups, so that standardisation of the method is clearly needed. There is also a need for improved measurement equipment allowing easy use of different exhalation flow rates without the need for the patient to maintain a steady exhalation effort.

6.4 Single or Multiple Flow Rates in Exhaled NO Measurement?

Currently, the exhaled NO concentration is recommended to be measured at a single exhalation flow rate of 50 ml/s (American

Thoracic Society 1999, Baraldi and de Jongste 2002). This method can be used to detect pulmonary inflammation, but it cannot differentiate between the bronchial and alveolar components in the inflammatory process. The single flow rate method is also insensitive to alveolar / peripheral inflammation (see chapter 6.1). However, the results of the present study and those obtained by other groups show that application of the two-compartment model by measuring exhaled NO concentration at multiple flow rates provides the advantage of differentiating between alveolar and bronchial components in pulmonary inflammation (Jörres 2000, Högman et al. 2000, Högman et al. 2001, Delclaux et al. 2002). If only higher flow rates (> 50 ml/s) are used, the exhaled NO concentration can be partitioned into alveolar NO concentration and bronchial NO flux (Tsoukias and George 1998). However, if also very low flow rates are included, bronchial NO flux can further be divided into its two components: bronchial wall NO concentration and bronchial diffusing capacity of NO (Högman et al. 2000, Silkoff et al. 2000). Different methods of exhaled NO measurement according to flow rates used are compared in Table 2.

In addition to the results presented in this thesis, also other study groups have applied exhaled NO measurement at multiple flow rates to different pulmonary conditions. The results are summarised in Table 3. Increased bronchial NO flux but normal alveolar NO concentration has consistently been found in asthma, which is in line with increased exhaled NO concentration at a single flow rate. In addition, in the present study increased alveolar NO concentration was found in nocturnal asthmatics and subjects with asthmatic symptoms but normal lung function. Although asthmatics can be differentiated from healthy subjects by measuring the exhaled NO concentration at a single flow rate, this peripheral component of asthmatic inflammation cannot be found.

Patients with FASSc have been found to have increased alveolar NO concentration, which is in line with the results on alveolitis in this work. Increased NO production has also been found in FASSc by measuring the exhaled NO concentration at a single flow rate (Paredi et al. 1999), but, again, the inflammatory process cannot be anatomically located by the single flow rate

Table 2. Gained parameters and information by exhaled NO measurement at different techniques according to the exhalation flow rates used.

Exhalation flow rates used	Parameters gained	Information gained
Single flow rate	-Exhaled NO concentration	-Presence / absence of pulmonary inflammation (not sensitive for alveolar/peripheral inflammation)
Multiple relatively high flow rates (> 50 ml/s)	-Alveolar NO concentration, -Bronchial NO flux	-Presence / absence of pulmonary inflammation, -Differentiation between alveolar and bronchial inflammation
Multiple flow rates, both low and high	-Alveolar NO concentration, -Bronchial wall NO concentration, -Bronchial diffusing capacity of NO	-Presence / absence of pulmonary inflammation, -Differentiation between alveolar and bronchial inflammation, -Whether changes in bronchial NO flux are due to changes in tissue concentration or diffusion of NO

method.

Shin and colleagues estimated parameters of alveolar and bronchial NO dynamics in children with cystic fibrosis and in healthy children (Shin et al. 2002b) by measuring exhaled NO with a new application of the two-compartment model utilising a single exhalation with dynamically decreasing flow rate (Tsoukias et al. 2001b). They found an increased bronchial wall diffusing capacity of NO, but a decreased alveolar NO concentration and bronchial wall NO concentration in cystic fibrosis. The authors attribute these changes to increased chemical consumption of NO by e.g. superoxide, whose production is increased in cystic fibrosis. The increased chemical reaction rate of NO in the tissue phase would reduce the tissue concentration of NO, this explaining the decreased C_W and C_{Alv}. An increased reaction rate of NO would also alter the tissue concentration

TABLE 3. Alveolar and bronchial NO parameters in different pulmonary diseases as compared with healthy subjects.

Disease state	C_{Alv}	$J_{NO,Br}$	C_W	$D_{NO,Br}$	Reference
Asthma		⇒	⇐	⇒	Silkoff et al. 2000
	⇐	⇒			Lehtimäki et al. 2000 (I) & 2001 (II)
	⇐	⇒			Högman et al. 2001
	⇐	⇒			Delclaux et al. 2002
	⇐		⇒	⇒	Högman et al. 2002b
Nocturnal asthma	⇒	⇒			Lehtimäki et al. 2002 (IV)
Asthma-like symptoms*	⇒	⇒			Lehtimäki et al. 2003 (V)
CFA & EAA	⇒	⇐			Lehtimäki et al. 2000 (I) & 2001 (II)
FASSc	⇒	⇔			Girgis et al. 2002
Smoking	⇐	⇔			Delclaux et al. 2002
	⇐		⇔	⇐	Högman et al. 2002b
	⇒	⇔			Högman et al. 2002a
COPD	⇒		⇐	⇐	Högman et al. 2002b
Cystic fibrosis	⇔	⇐	⇔	⇒	Shin et al. 2002

C_{Alv}, alveolar NO concentration; $J_{NO,Br}$, bronchial NO flux; C_W, bronchial wall NO concentration; $D_{NO,Br}$, bronchial wall diffusing capacity of NO; CFA, cryptogenic fibrosing alveolitis; EAA, extrinsic allergic alveolitis; FASSc, fibrosing alveolitis associated with systemic sclerosis; *, patients with asthmatic symptoms but normal lung function; ⇐, not different from healthy subjects; ⇒higher than in healthy subjects; ⇔lower than in healthy subjects.

profile of NO in the bronchial wall, leading to an increased bronchial wall diffusing capacity of NO. This together with a decreased tissue concentration of NO would tend to balance out each other's effects on bronchial NO flux. The slightly decreased alveolar NO concentration and fairly normal bronchial NO flux would lead to normal or slightly decreased exhaled NO concentration at a single flow rate. Thus, patients with cystic fibrosis cannot necessarily be distinguished from healthy subjects by exhaled NO measurement at a single flow rate, although considerable changes in pulmonary NO dynamics can be shown by separately assessing the alveolar and bronchial NO parameters (Shin et al. 2002b). This example again nicely indicates the superiority of the extended NO measurements over the single flow rate method.

Högman and colleagues have reported an increased bronchial wall diffusing capacity of NO also in patients with allergic rhinitis without asthma (Högman et al. 2002b). On the other hand, the bronchial wall NO concentration in these patients was normal. The results suggest that the increased exhaled NO concentration found in allergic rhinitis (Henriksen et al. 1999, Gratziou et al. 2001) is caused by an increased bronchial mucosal area producing NO rather than by increased intensity in NO production.

Delclaux and colleagues have found increased alveolar NO concentrations but normal bronchial NO output in patients with liver cirrhosis (Delclaux et al. 2002). The hepatopulmonary syndrome caused by liver cirrhosis is associated with increased vascular endothelial NO production (Albillos et al. 1995) and increased iNOS expression in macrophages in pulmonary capillaries (Nunes et al. 2001), which presumably explains the increased alveolar NO concentration in cirrhotic patients. Increased exhaled NO concentrations in liver cirrhosis have also been shown at a single flow rate (Cremona et al. 1995, Matsumoto et al. 1995, Sogni et al. 1995) but, again, these measurements cannot indicate where in the lung NO dynamics is altered.

Smoking has been shown to reduce bronchial NO parameters (Delclaux et al. 2002, Högman et al. 2002b), and one study also shows increased alveolar NO concentration as an early sign of smoking-induced pulmonary damage (Högman et al. 2002a). The decreased bronchial NO flux in smokers is consistent with the decreased exhaled NO concentration at single flow rate, but the single flow rate method might not be useful in detecting the increased alveolar NO concentration in these subjects.

Högman and colleagues also report increased alveolar NO concentrations but normal bronchial NO parameters in patients with COPD (Högman et al. 2002b). This increased alveolar NO concentration in COPD might reflect a decreased alveolar diffusing capacity of NO due to emphysema or increased alveolar NO production associated with inflammation. The exhaled NO concentration at a low exhalation flow rate reflects bronchial NO dynamics, while the contribution from the alveolar level increases with increasing flow rate. This might explain why COPD has been associated with increased exhaled NO concentrations in studies using a single higher flow rate (– 100 ml/s) (Maziak et al.

1998, Paredi et al. 2000) but with normal exhaled NO levels in studies using a single lower flow rate (– 50 or 42 ml/s) (Kanazawa et al. 1998, Ichinose et al. 2000).

The difference between measuring exhaled NO at a single or at multiple flow rates can be compared to the difference between measuring peak expiratory flow (PEF) or spirometric flow-volume curves. PEF measurements are obviously of clinical value in detecting impaired lung function, but the parameters obtained from flow-volume spirometry are more sensitive and give more detailed information on airway function. Similarly, exhaled NO measurement at a single flow rate can be used to detect pulmonary inflammation, although it is not sensitive to peripheral inflammation at the currently recommended flow rate (50 ml/s), but NO measurement at multiple flow rates gives more detailed information of pulmonary NO dynamics and on the underlying inflammatory process. It is therefore likely that the multiple flow rate method will displace the single flow rate method. International guidelines on exhaled NO measurement should be revised to include the multiple flow rate method, which significantly improves the clinical value of exhaled NO measurement.

There have been several different applications of the multiple flow rate method. Some have used only higher flow rates, others also include very low flow rates, and a dynamically changing flow rate during a single exhalation has also been used. Further studies are needed to assess how the results are affected by the number and magnitude of chosen flow rates, and whether the flow rates are to be achieved during a single or multiple exhalations.

7. SUMMARY AND CONCLUSIONS

Currently the diagnosis and follow-up of inflammatory lung diseases are largely based on radiography and measures of lung function to detect secondary changes caused by the inflammatory process. There is a need to develop methods allowing direct measurement of pulmonary inflammation. The present study focused on calculating alveolar NO concentration and bronchial NO flux based on measuring the exhaled NO concentration at multiple exhalation flow rates. The aim was to test whether assessment of alveolar NO concentration and bronchial NO flux could be used to differentiate between alveolar and bronchial components in inflammation, and to establish whether alveolar and bronchial NO output correlate with disease severity and respond to anti-inflammatory treatment in asthma and alveolitis. Alveolar and bronchial NO output were also studied in nocturnal asthma and in patients with asthmatic symptoms but normal lung function (asthma-like syndrome). The summary and conclusions of the main results of the present study are as follows:

1. Patients with asthma had increased bronchial NO flux but on average normal alveolar NO concentration, whereas patients with alveolitis had increased alveolar NO concentration but normal bronchial NO flux. In asthma, increased bronchial NO flux correlated with markers of eosinophilic inflammation and bronchial hyperresponsiveness. In alveolitis, increased alveolar NO concentration correlated negatively with pulmonary diffusing capacity and alveolar volume. It is concluded that measurement of the alveolar NO concentration and bronchial NO flux by the multiple flow rate method can be used to differentiate between alveolar and bronchial inflammation and to assess disease severity in asthma and alveolitis.

2. Treatment of asthma with inhaled glucocorticoids reduced the bronchial NO flux back to normal while alleviating symptoms and improving lung function. Anti-inflammatory treatment in alveolitis lowered the alveolar NO concentration towards normal and improved the pulmonary diffusing capacity. It is concluded that measurement of alveolar NO concentration and bronchial NO flux can be used to study the efficacy of anti-inflammatory treatment in alveolitis and asthma, respectively.

3. Consistently with the intensified alveolar inflammation reported in nocturnal asthma, asthmatic patients with nocturnal symptoms had higher alveolar NO concentrations but bronchial NO flux similar to that in asthmatics with only daytime symptoms. It is concluded that measurement of the alveolar NO concentration can be used to detect the peripheral component in asthmatic inflammation associated with nocturnal asthma.

4. Subjects with asthmatic symptoms but normal lung function (asthma-like

syndrome) had slightly increased bronchial NO flux and also enhanced alveolar NO concentration. It is concluded that measurements of lung function are not sufficient to detect all patients with airway inflammation, but a direct measure of inflammation should be used to identify those with an inflammatory process underlying their asthma-like symptoms. The results also suggest that this subject group had more peripheral airway inflammation than patients fulfilling the current diagnostic criteria for asthma.

The results of the present study series show that the multiple flow rate method in exhaled NO measurement can be used to assess the presence and severity of alveolar and bronchial components in pulmonary inflammation separately in a non-invasive manner. Alveolar / peripheral inflammation is not detectable by exhaled NO at a single low flow rate, while at a single high flow rate it cannot be differentiated from bronchial inflammation. The multiple flow rate method thereby gives clinically more useful information on pulmonary inflammation than the single flow rate method of exhaled NO measurement. Future studies should seek to provide information on the applicability of the multiple flow rate method to other pulmonary diseases. There is also a need to standardise the method by studying which and how many flow rates should be used, and to ascertain what is the additional clinical value of dividing bronchial NO flux further into its components (bronchial wall NO concentration and NO diffusing capacity). Measuring equipment allowing easy use of different exhalation flow rates should be developed to facilitate research and clinical practice in the field.

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APPENDIX 1

Kyselykaavake astmaoireista

Astman tyypillisiä oireita ovat mm. yskä, hengityksen ajoittainen vinkuminen ja hengenahdistus. Alla esitetään neljä astmaoireita koskevaa kysymystä, joihin jokaiseen on neljä vastausvaihtoehtoa. Ympyröikää niistä Teidän tilannettanne viimeisen viikon aikana parhaiten kuvaava vaihtoehto.

1. Onko Teillä viimeisen viikon aikana ollut yskää?

   0 Ei ollenkaan.
   1 Yhtenä päivänä.
   2 Kahtena tai useampana päivänä.
   3 Joka päivä.

2. Onko Teillä viimeisen viikon aikana ollut hengityksenvinkumiskohtauksia?

   0 Ei ollenkaan.
   1 Yhtenä päivänä.
   2 Kahtena tai useampana päivänä.
   3 Joka päivä.

3. Onko Teillä viimeisen viikon aikana ollut hengenahdistusta?

   0 Ei ollenkaan.
   1 Yhtenä päivänä.
   2 Kahtena tai useampana päivänä.
   3 Joka päivä.

4. Ovatko astmaoireet (yskä, hengenahdistus tai hengityksen vinkuminen) haitanneet nukkumistanne viimeisen viikon aikana?

   0 Ei ollenkaan.
   1 Yhtenä yönä.
   2 Kahtena tai useampana yönä.
   3 Joka yö.

English translation of the asthma symptom questionnaire

Cough, wheezing of breath and chest tightness are typical symptoms of asthma. Below are four questions concerning asthma symptoms, each having four optional answers. Please choose the answer option on each point that best describes your symptoms during the last week.

1. Have you had cough during the last week?

   0 Not at all.
   1 On one day.
   2 On 2 or more days.
   3 Every day.

2. Have you had wheezing of breath during the last week?

   0 Not at all.
   1 On one day.
   2 On 2 or more days.
   3 Every day.

3. Have you had chest tightness during the last week?

   0 Not at all.
   1 On one day.
   2 On 2 or more days.
   3 Every day.

4. Have symptoms of asthma (cough, wheezing of breath, or chest tightness) disturbed your sleep during the last week?

   0 Not at all.
   1 On one night.
   2 On 2 or more nights.
   3 Every night.

ORIGINAL COMMUNICATIONS