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Nasal airway nitric oxide -

methodological aspects and influence of inflammation

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“Inga extranummer!”
Socker Conny, by Joakim Pirinen.

To Katarina Barklund Palm
and Christina Barklund

ABSTRACT

Nitric oxide (NO) is an endogenously formed free radical gas involved in numerous biological processes. In 1991 NO was discovered to be present in exhaled air of humans. Soon after, it was reported that the largest amounts of NO were found in the upper airways, and that the levels of NO were increased in the lower airways of patients with asthma. The high levels of NO in the nasal region are believed to be involved in functions as various as primary host defence, including killing of microbes and stimulation of ciliary motility, in inflammation and in aiding oxygen uptake in the lower airway. Extremely low values of nasal NO have been found in patients with ciliary dysfunction and cystic fibrosis. Consensus has been reached on how to measure orally exhaled NO, but the methodology for nasal NO measurements is still being discussed.

Besides NO, carbon monoxide (CO) is also found in exhaled air, and, like NO, CO levels are altered in various airway disorders. Furthermore, CO has been found in the upper airways of healthy humans. Thus, CO and NO seem to coexist in the airways, both in health and disease.

We wanted to establish a new method for nasal measurements of NO and CO, and to characterize normal upper and lower airway output of NO and CO in healthy subjects. We also wanted to study airway NO release in humans with systemic or localized inflammation, as in HIV/AIDS, endotoxaemia and allergic rhinitis.

Surprisingly, we found that only NO, but not CO, could be consistently detected in the healthy human upper airway. We also found that a mouthwash procedure, aiming at increasing pH in the oral cavity, did not influence levels of nasally exhaled NO, whereas it reduced both oral contribution to and methodological variation in the measurements of NO in orally exhaled air. Hence, we introduced a new method for nasal NO measurements, based upon a highly standardized single breath technique The method provides information about the contribution of the supravelar space to airway NO release.

Using this method, we found reduced nasal NO levels in patients with HIV/AIDS, and suggested that this reduction may contribute to the decreased resistance to airway infections in these patients.

Orally exhaled NO, but not nasal NO, increased during experimental human endotoxaemia. Further studies will show whether exhaled NO may be valuable as a marker of sepsis-induced lung injury.

Also, a high inter-individual variation in nasal NO levels was found, and orally exhaled NO levels were elevated in patients with allergic rhinitis. Further studies will reveal if the patients with allergic rhinitis and decreased nasal NO are at risk of developing paranasal sinus disease, and if those with increased orally exhaled NO are at risk of developing asthma.

In conclusion: Nasal NO measurements are non-invasive and easy to perform. With improved methodology such measurements may be useful for the screening, diagnosing or monitoring of inflammatory disorders affecting the upper airways.

Key words: Asthma, breath test, exhaled air, rhinitis, sinuses, sinusitis, diurnal variation, gender, nasal, oral, nitric oxide, volume flow rate, AIDS, HIV, endotoxin, gut, human, shock, allergy.

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referenced by their Roman numerals:

I Nitric oxide but not carbon monoxide is continuously released in the human nasal airways. J.O. Lundberg, J. Palm, K. Alving. European Respiratory Journal 2002; 20: 100-103.

II Characterization of exhaled nitric oxide: Introducing a new reproducible method for nasal nitric oxide measurements. J.P. Palm, P. Graf, J.O. Lundberg, K. Alving. European Respiratory Journal 2000; 16: 236-241.

III Reduced nasal nitric oxide in patients with HIV. J. Palm, C. Lidman, P. Graf, K. Alving, J.O. Lundberg. Acta Otolaryngol 2000; 120: 420-423.

IV Exhaled NO and plasma cGMP increase after endotoxin infusion in healthy volunteers. A. Soop, A. Sollevi, E. Weitzberg, J.O. Lundberg, J. Palm, J. Albert. European Respiratory Journal 2003; 21: 1-6.

V Characterization of airway nitric oxide in allergic rhinitis: The effect of intranasal administration of L-NAME. J.P. Palm, K. Alving, J.O. Lundberg. Allergy 2003: 58: 885-892.

ABBREVIATIONS

AR	allergic rhinitis
AP-1	activator protein-1
cGMP	cyclic guanosine monophosphate
CI	confidence interval
cNOS	constitutive nitric oxide synthase
CF	cystic fibrosis
CO	carbon monoxide
CO_2	carbon dioxide
ERDF	endothelium-derived relaxing factor
eNOS (NOS III)	endothelial nitric oxide synthase
HO-1	haeme oxygenase-1
HO-2	haeme oxygenase-2
IL-1	interleukin-1
IFNγ	interferon gamma
iNOS (NOS II)	inducible nitric oxide synthase
IR	infrared
L-NAME	N^G-nitro-L-arginine methyl ester
LPS	lipopolysaccharide
NDIR	non-dispersive infrared
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
nNOS (NOS I)	neuronal nitric oxide synthase
PCD	primary ciliary dyskinesia
ppm	parts per million
ppb	parts per billion
SD	standard deviation
SEM	standard error of the mean
TLC	total lung capacity
TNFα	tumour necrosis factor α
URTI	upper respiratory tract infection

CONTENTS

INTRODUCTION

Nitric oxide (NO) is a free radical gas formed in the atmosphere, e.g. during thunderstorms and during the combustion of organic materials. Studies on the interaction between nitrogen oxides and humans have been carried out for many years. For example in a work from 1865, Hermann describes spectroscopic studies on blood revealing that NO combines reversibly with haemoglobin, and, notably, that haemoglobin has higher affinity for NO than for carbon monoxide (CO), and therefore much greater than for oxygen. That work also mentions how, in the haze ("in einem entusiastischen Augenblick") of having inhaled NO-gases, one scientist by the name of Davy attempted to inhale nitrogen dioxide (NO_2) in order to free the airway of oxygen. Fortunately he did not succeed (1). It was Mr. Davy who in 1800 suggested the use of nitrous oxide (N_2O), billed as "laughing gas", to relieve the pain of surgery (2). Long thought of as merely one of the toxic or noxious gases in the nitrogen oxides complex (NO_X), NO was not the focus of much research activity until the 1980's, when NO was discovered to be endogenously formed in mammals, and to be involved in numerous biological processes of great importance.

The understanding of cell-cell communication was at the time perhaps dominated by the paradigm of signalling being accomplished by molecules binding to specific receptors through complementarity of shape. NO research led to further understanding of a system where the messenger reacts with its targets on the basis of their redox potential (3). Notably, the discoveries on NO and vascular regulation, identifying NO as the endothelium-derived relaxing factor (ERDF) needed for relaxation of smooth muscle cells and consequent vasodilatation, led to the Nobel prize in Physiology or Medicine in 1998 (4-6). It is perhaps worth mentioning that these insights cast new light on the effect of the ingested explosive nitroglycerin. Nitroglycerin acts as an NO donor and therefore relieves angina pectoris, from which Mr. N himself is said to have suffered from. In 1992 NO was named "molecule of the year" by the journal Science (7).

Chemistry of NO

NO is a colourless gas with a weight of only 30 U. Indeed, NO has one of the lowest molecular weights of any known bioactive product secreted by mammalian cells. Due to its small size, lipid solubility and uncharged nature it readily diffuses across biological membranes. The presence of an unpaired electron makes NO a radical, a fact sometimes emphasized by the addition of a dot ($NO^{\bullet}$) (3). In air, NO reacts with oxygen to form nitrogen dioxide ($2NO + O_2 \rightarrow 2NO_2$), a brown gas capable of inducing severe tissue damage. In biological systems, NO is usually unstable due to reactions with a variety of compounds, including metals, thiol-groups or other radicals. NO reacts rapidly, e.g. with the reactive oxygen species (ROS) superoxide to form the potent oxidant peroxynitrite ($NO + O_2^- \rightarrow OONO^-$). However, at low concentrations in gas phase, NO is fairly stable, even in the presence of oxygen (8, 9). In the lung, NO uptake is mainly determined by its high diffusion capacity, the reaction with intravascular oxyhaemoglobin, whereby it is oxidised to nitrate, and by another local reaction in the airway lining fluid yielding bioactive S-nitrosoglutathione (9).

Briefly, in biological systems, NO is inactivated by combination with the haem of haemoglobin or by oxidation to nitrite (NO_2^-) and then nitrate (NO_3^-) (10). In water, ultrafiltrate, and plasma, NO is oxidised to nitrite, which is stable for several hours. In whole

blood, however, nitrite is rapidly converted to nitrate, which is excreted in the urine. Thus, basal nitrite concentrations in blood are low, and those of nitrate are about 100 times higher (approximately 30 μmol per liter) (8).

NO synthesis

Outside of biological systems, NO is produced from its constituent elements N_2 and O_2, mainly at elevated temperatures. Biosynthesis, on the other hand, relies on other factors.

-Nitric oxide synthases

Three isoforms of NOS can be differentiated in mammalian species: neuronal NOS (nNOS or type I NOS), inducible NOS (iNOS or type II NOS) and endothelial NOS (eNOS or type III NOS). In humans, these enzymes are genetically coded on chromosomes 12, 17 and 7, respectively. The constitutively expressed NOS I and III, which release NO in the picomolar range are Ca^{2+}-dependent, whereas NOS II, capable of releasing thousand-fold larger amounts of NO, is not (11). L-arginine, O_2 and nicotinamide adenine dinucleotide phosphate (NADPH) are co-substrates; flavin mononucleotide (FMN), flavin adenin dinucleotide (FAD), haem and tetrahydrobiopterin are cofactors; and NO and citrulline are co-products of the reaction catalysed by NOS (3).

-Regulation of enzymatic NO production

A quintessential distinction between different forms of NOS consists in whether they bind calmodulin in a reversible, Ca^{2+}-dependent manner and hence are susceptible to activation by agonists that transiently elevate Ca^{2+}, or instead are regulated by induction of transcription. Glucocorticoids inhibit the induction of the Ca^{2+}-independent NOS but not the activity of either the constitutive or the inducible enzyme. iNOS is also highly regulated by LPS and cytokines, of which some (e.g. IFNγ, TNFα and IL-1) induce and others (e.g. TGFβ, IL-4, IL-10) inhibit expression of the enzyme (3, 8). Also, iNOS may be activated by the transcription factors NF-κB and AP-1 (12).

The first described NOS inhibitor, the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA), has been used to identify many of the physiological actions of NO. Another widely used NOS inhibitor is N^G-nitro-L-arginine methyl ester (L-NAME) (13).

-NOS independent NO production

Apart from enzymatic NO synthesis, another pathway for NO formation was recently described (14, 15). At a low pH, nitrite (NO_2^-) will form nitrous acid (HNO_2^-), which subsequently decomposes to various nitrogen oxides including NO. The rate of which the reactions occur and which way they are driven depend on ambient conditions including pO_2, pH, proximity to haem-containing proteins, redox state and thiol concentrations. In the human stomach, high levels of NO are found, leading to the suggestion that NO is involved in gastric host defence, regulation of mucosal blood flow and mucus generation (16). The nitrite may come from dietary intake or from saliva, through reduction of nitrate to nitrite by bacteria in

the oral cavity. Besides in the human stomach and oral cavity, this non-enzymatic NO production has been demonstrated in urine, heart, on the skin and in ischaemic tissues (17).

In the airways, orally exhaled NO, but not nasal NO has been shown to increase following ingestion of nitrate, indicating that the non enzymatic production in the airways occurs in the oral cavity (18).

Biological roles of NO

The biological effects of NO are extremely diverse, ranging from regulation of vascular tone, and platelet function to neurotransmission, penile erection and killing of pathogens. NO exerts many of its biological actions through the activation of intracellular cytosolic soluble guanylyl cyclase (sGC) and the generation of cyclic guanosine monophosphate (cGMP). The message carried by cGMP varies with the tissue in which it acts. Most of the actions of cGMP are believed to be mediated by cGMP-dependent protein kinase, also called protein kinase G (19). Haem-deficient guanylyl cyclase is unresponsive to NO, whereas the haem-containing forms are activated up to 100-fold by NO (20).

NO generated by eNOS in vascular endothelial cells, for example upon stimulation by shear stress or acetylcholine, maintains vasodilator tone through the relaxation of smooth muscle cells, and inhibits platelet aggregation.

Neuronal NOS (nNOS), present in the central nervous system, is involved in the transduction mechanisms triggered when glutamate activates its receptor. Glutamate receptors play vital physiologic roles such as formation of memory, vision, feeding behaviour, nociception and olfaction. In the periferal nervous system nNOS is involved in nonadrenergic, noncholinergic nerve transmission and regulation of e.g. tone in the bronchi and in the corpus cavernosum.

Host defence and inflammation are functions mainly ascribed to iNOS, present in white blood cells and other cells. Expression of iNOS is induced e.g. by LPS, IFNγ, IL-1 or TNFα. This induction, which is inhibited by glucocorticoids, results in the sustained production of NO, which diffuses to target cells such as tumour cells, bacteria, fungi and helminths (8).

All three enzymes have been found in the human airways. Constitutive NOS is expressed in neuronal cells (NOS I), endothelial cells (NOS III) and epithelial cells (NOS I and NOS III). NOS II expression has been described in epithelial cells, macrophages, neutrophils, endothelial cells and vascular smooth muscle cells. However, the predominant form in airway epithelium is the inducible NOS (NOS II) (21). Where autonomic innervation of human airways is concerned, the inhibitory nonadrenergic, noncholinergic (iNANC) nerves may be the only neural bronchodilator pathways, and NO appears to be the major neurotransmitter of these iNANC nerves (22).

Measurement of NO

Due to the short halflife of NO in most biological systems, NO assay methods are often based upon indirect measurements on products of NO metabolism. However, as mentioned, NO is fairly stable in the gaseous phase, enabling direct measurements, e.g. by chemiluminescence.

Besides this highly sensible technique for the identification of NO, one may turn to mass spectrometry, electrochemical measurements and electron paramagnetic resonance. Assays for the measurement of expression, activity and cofactor consumption of NOS include NOS immunohistochemistry, NADPH diaphorase staining and citrulline assay. Analythical methods for detection of the stable oxidation products nitrite and nitrate include the Griess assay, capillary electrophoresis and high pressure liquid chromatography (9).

Carbon monoxide (CO)

CO is an inert gas found in environmental toxic fumes. It is also produced endogenously in humans and found in expired breath, with elevated levels in smokers (23). Haeme oxygenase (HO) is the rate-limiting enzyme in the degradation of haeme, producing biliverdin, iron and CO, and two isoforms have been characterized. The first is the inducible HO-1, which responds to many types of stimuli having in common the ability to cause oxidative stress, and the other is the constitutively expressed HO-2, which seems responsive only to adrenal glucocorticoids. Like NO, CO has been reported to exert biological actions through the activation of soluble sGC and generation of cGMP. CO signalling has been reported in the nervous system, the reproductive system, the cardiovascular system, liver and spleen, and the HO system seems involved in e.g. memory formation, spermatogenesis, response to oxidative stress and vascular perfusion (12). Induction of HO-1 by reactive species followed by an increased CO production has been put forward as a general cytoprotective mechanism against oxidative stress (12) and therefore potentially clinically useful in detection and management of inflammatory lung disorders (24). In fact, CO itself is suggested to have antioxidant, cytoprotective and anti-apoptotic effects based on results from studies on the administration of exogenous CO in various experimental settings (25).

Relation between NO and CO system?

It is becoming increasingly evident that regulation of HO and NO production by NOS:s are intimately linked. The common purpose for both NO and CO could be regarded as maintenance of basal cGMP production.

Because NOS is a haemoprotein, it would be subject to all factors that affect a haemoprotein's stability, synthesis and turnover. As mentioned, HO is the rate-limiting enzyme in the degradation of haem. During inflammation, when iNOS is induced, resulting in high NO production and oxidative stress, HO-1 will be induced and thus increase the capacity to degrade the very substrate for further iNOS formation. Notably, this occurs at the same time as the antioxidants bilirubin and ferritin are formed from biliverdin and iron, respectively (25). Thus, the induction of HO-1 could be considered a cellular defence mechanism. This enhanced production of CO is readily measurable in airways (24). Under normal conditions, then, HO-2 would function in maintaining the basal cGMP production alone or together with

NOS, depending on the cell and tissue. Once NO has bound to an enzyme, regulating its action, it dissociates at an extremely slow rate. The HO system has also been suggested to fulfil the role of making it possible to rapidly terminate the regulatory action of NO. Furthermore, nuclear factor (NF)-κB and activator protein (AP)-1 are transcriptional factors that are activated in the presence of oxygen free radicals, and both HO-1 and iNOS have NF-κB and AP-1 regulatory elements in their promoter regions (12).

NO in host defence and inflammation

Both NO formation and iNOS are up-regulated in inflammatory conditions. Other than in asthma (26), increased NO production has been described in several inflammatory disorders such as rheumatoid arthritis (27), sepsis (28), cystitis (29) and colitis (30). When iNOS is induced, large amounts of NO can indeed be released, not only by white blood cells but also from epithelium. The role of this NO is not entirely clear in that it may have both beneficial and detrimental effects.

Judging from the very high concentrations of this gas normally found in the stomach (14, 31) and in the paranasal sinuses (32, 33), NO does not seem harmful per se. However, interaction of NO with oxygen free radicals that are normally generated in the course of cellular oxygen metabolism can lead to the formation of other nitrogen radicals – e.g. peroxynitrite, nitrogen dioxide - and hydroxyl radicals, all of which are much more toxic than NO itself. These species are suspected to be involved in target cell injury but also in the killing of pathogens (12). The biochemical basis for the cytotoxicity induced by NO and related products may be the combination of these compounds with iron-containing moities in key enzymes of the respiratory cycle and of DNA synthesis in the target cells (20).

The major oxidants in airways are reactive oxygen and reactive nitrogen species (ROS/RNS). Airway disease such as asthma, cystic fibrosis and chronic obstructive pulmonary disease (COPD) are all associated with oxidative stress (34, 35). And even though HO-1 is considered one of nature's main defences against oxidizing environment (25), endogenous CO may also become harmful under chronic stress combined with oxidative stress. If adrenal glucocorticoids are increased, HO-2 expression and – subsequently - CO production increase. Excess CO will compete with oxygen to bind to haemoglobin, causing oxidative stress. The ensuing induction of HO-1 may then further increase the cellular burden of CO. This combined increased CO generating capacity would lead to influx of Ca^{2+} and not only activation of NOS and subsequently more NO, but also to apoptotic cell death (12).

Human airway NO

In 1991 Gustafsson and co-workers found that NO was present in exhaled air of rabbits, guinea pigs and humans (36). In 1993 it was reported that the high levels of NO in human airway are found in the upper airways, and that NO levels are increased in the lower airways of patients with asthma (26). NO released from the nasopharynx and then inhaled was shown to be resorted by the lower respiratory tract, and symbiosis regarding bacterial and upper airway NO production was suggested (37). Also, inhaled exogenous NO was shown to reverse pulmonary hypertension, presumably by acting as a selective pulmonary vasodilator (38, 39).

These findings triggered a great interest to study NO in the airways, both as a potential diagnostic marker (exhaled NO) and as a therapeutic agent (inhaled NO).

Nasal airway NO

In 1994/95 it was reported that the highest levels of NO produced endogenously in the airways were to be found in the paranasal sinuses, released by what resembled a continuously-expressed inducible nitric oxide synthase (iNOS) (32, 33). The concentrations found were equivalent to those considered toxic in the outdoor environmen (25 ppm) (40). At the same time it was shown that nasal inhalation of these high concentrations of NO improved oxygenation, suggesting that endogenously produced upper airway NO acts as an “aerocrine messenger in humans” (41, 42). Interestingly, patients with ciliary dysfunction, characterized by immotility of spermatozoa and respiratory cilia, along with chronic oto-sino-pulmonary disease (43), sometimes associated with situs inversus (44), showed almost a total lack of nasal NO (45). A role for NO in primary host defence in the upper airways has been proposed (33). Notably, even very low concentrations of NO (<1ppm) have shown bacteriostatic activity against various bacteria, including Staphylococcus aureus (46). NO has also been suggested to play an important role in the up-regulation of ciliary motility (47), and low levels of nasal NO have been shown to correlate with impaired mucociliary function in the human upper airway (48).

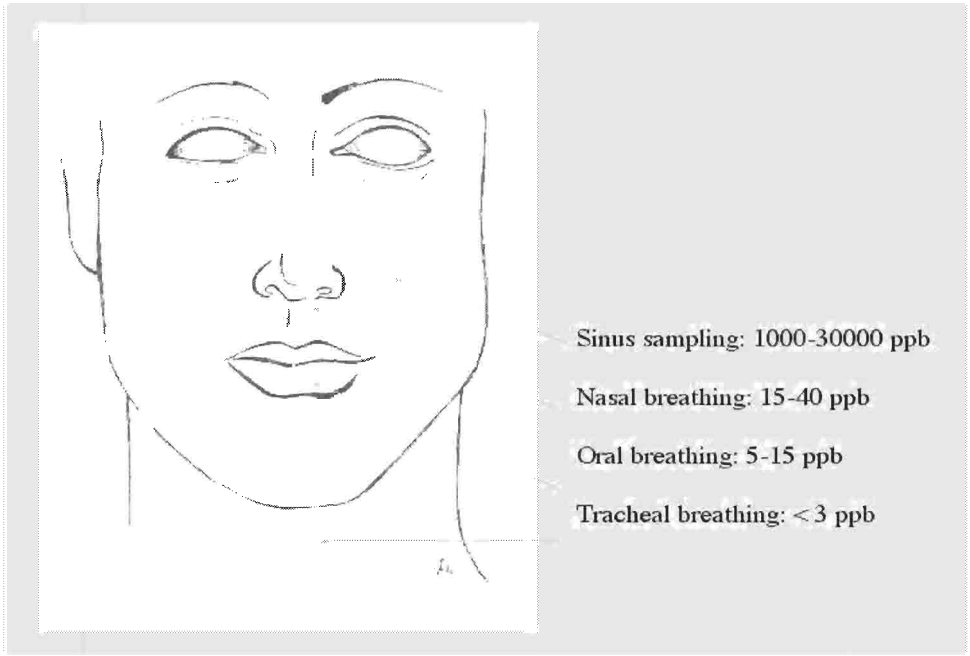

Figure 1. Typical levels of NO found in human healthy airway.

Airway NO in inflammation

Since measurements of airway NO are non-invasive, but nonetheless give quick information reflecting local production of this gas, there has been a great interest to study if NO measurements could be of use clinically to diagnose airway disease and to monitor therapy.

-Exhaled NO

Asthma is an inflammatory disease leading to hyper-responsiveness and variable obstruction of the airways, and characterised by influx of inflammatory cells and mediators, along with epithelial damage. To make a diagnosis, the physician may rely on clinical history and examination, but other methods for diagnosis and clinical evaluation, such as spirometry, skin-prick test, serological tests, induced sputum, airway hyperresponsiveness and bronchoalveolar lavage, may provide further information. NO, as above mentioned, is known to be a mediator of vasodilatation and bronchodilatation. The existence of iNOS in lung biopsies from human asthmatics has been reported and NO has been proposed to be relevant to the pathology of asthma (49). Increased NO has been widely documented in exhaled air of asthmatics (26, 50-55), and the levels of NO seem to decrease dose-dependently after anti-inflammatory treatment with glucocorticoids (52-54). Significant correlations between exhaled NO, the concentration of methacholine required to produce a 20% drop in FEV_1, and sputum eosinophils have been found in asthmatics (56). However, airway calibre, smoking status and previous alcohol ingestion should be taken into account when interpreting these measurements (57). Standardized methods for the measurement of orally exhaled NO have

been published, both by the American Thoracic Society and the European Respiratory Society (58, 59) and exhaled NO is now close to introduction in clinical practice in several countries. Apart from in asthma, increased levels of exhaled NO have been found in endotoxaemia (60), unstable COPD, bronchiectasis, fibrosing alveolitis, and laboratory animal allergy, whereas levels seem decreased in cystic fibrosis, pulmonary hypertension/cor pulmonale, interstitial lung disease and HIV (61).

-*Nasal NO*

In view of all knowledge achieved through studies on NO, it is tempting to evaluate further the influence of nasal airway NO in man. The nose, the very port of the airway at rest, and the very main inlet of airway NO in health.

For the management of patients with rhinitis the physician may rely on clinical history and examination. Objective methods such as skin-prick test, serological tests, peak inspiratory airflow, nasal airway resistance and acoustic rhinometry before and after nasal decongestants may provide additional information. For differential diagnosis or research purposes one may add biopsies, nasal lavage, rhinostereometry, saccharine testing, ciliary beat frequency and electron microscopy. If a method that could signal the level of ongoing airway inflammation could be found it could facilitate diagnosis and help optimize the treatment of these patients. Nasal NO levels in allergic rhinitis (AR) have been studied, but without any clear picture evolving. Some studies have pointed to an increase (62-64) whereas other have not been able to subscribe to this view (53, 65, 66). Notably, different methods for nasal NO measurements have been used in the studies. NO production in the nasal mucosa of patients with AR seems to be up-regulated according to histopathological studies on NOS (67-69). Apart from primay ciliary dyskinesia (PCD), decreased levels of NO have been found in patients with cystic fibrosis (CF) (53), active Wegener's granulomatosis (70), acute paediatric sinusitis (71) and chronic sinusitis in adults (72).

Airway CO

The recent years' great interest in airway NO research has triggered the search for other volatile gases that can be measured non-invasively for the benefit of patients with airway disease. As mentioned, CO is present in exhaled air and is increased in smokers (23). Also, CO in orally exhaled air is found to be increased in children (73) and adults with asthma (74), atopy (75), bronchiectasis (24), COPD (61), cystic fibrosis (76), and in allergic rhinitis (77). Moreover, in one study, high levels of CO were found in nasal and maxillary sinus air (78) of healthy subjects. Thus, CO and NO have been suggested to coexist in the upper and lower airways, both in health and disease.

AIMS OF THE STUDY

The overall aim of the study was to develop and use a novel method of measuring levels of endogenously produced gases in the human upper airways in health and disease, with particular interest in nitric oxide (NO) and carbon monoxide (CO).

Specific aims:

- To establish a new method for nasal measurements of NO and CO, and to characterize normal upper and lower airway output of NO and CO in healthy subjects.

- To study airway NO release in humans with systemic or localized inflammation: HIV/AIDS, endotoxaemia and allergic rhinitis.

MATERIALS AND METHODS

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

Analysis of NO and CO

- Gaseous NO (paper I-V)

These analyses were carried out using chemiluminescence technique (Aerocrine AB, Sweden). In brief, NO contained in the sampled air reacts with an excess of ozone (O_3) to produce NO_2 with an electron in an excited state (NO_2^*). This NO_2^* changes back to ground state while emitting electromagnetic radiation in the 600- to 3000-nm wavelength range. The chemiluminescence is detected by a photomultiplier tube that proportionally converts the intensity of luminescence into an electric signal for display. This technique is extremely sensitive and NO can be detected down to concentrations of approximately 1 ppb with no interference from other nitrogen oxides. This, combined with a response time for the system of <0.7 s, allows on-line measurements to be performed.

- Nitrite, nitrate and cGMP in plasma (paper IV)

Nitrite and nitrate concentrations in plasma were analysed with a fluorometric nitrate/nitrite assay kit according to the manufacturer's instructions. Cyclic GMP was assayed in plasma after ethanol extraction using a cGMP radioimmunoassay kit according to the manufacturer's instructions.

- Gaseous CO (paper I)

These analyses were carried out using infrared (IR) technique (UNOR 610; Maihak AG, Hamburg, Germany). In brief, non-dispersive infrared (NDIR) spectroscopy is based upon two equal infrared beams being directed through two parallel optical cells, one cell through which the sampled air is led and one reference cell. During analysis, CO absorbs a portion of the infrared radiation in the sample, the quantity absorbed being proportional to the CO concentration in the sample. A detector converts the difference in energy of the two beams to a capacitance change. The UNOR 610 has a response time of <3 s and a detection limit of 0.1 ppm, allowing on-line measurements to be performed.

Collection of gases for analysis

- Nasally aspirated NO and CO (paper I, V)

Nasal air was aspirated at various flow rates (0.5-9 l/min) from one nostril using a nasal olive. The analyser sampled air at a flow rate of 0.1 l/min, either directly from near the nasal olive or from a non-reactive polyurethane bag, to which the nasal air had been led. NO-free and CO-free air was introduced via a tightly fitting olive introduced in the contra-lateral nostril. During nasal measurements the subjects performed an oral single-breath exhalation against a resistance, or held their breath in order to obtain closure of the soft palate, all according to international recommendations (58, 59). In our studies, however, several air-flow-rates were used.

- Nasally and orally exhaled NO (paper I-V)

In brief, compressed NO-free air was continuously introduced into a non-diffusing gas collection bag. The bag was connected to a 3-way non-rebreathing valve which in turn was connected to an antiviral filter. This filter was directly used as a mouthpiece. The subjects were asked to inhale NO-free air via the mouth and immediately exhale via the same route, at various flow rates of air against various resistances, resulting in similar oral pressure. Exhaled air was continuously sampled, from a point close to the antiviral filter, and fed into the NO analyser. The signal outputs from these devices were connected to a computer-based system, yielding instant on-screen display of flow for biofeedback and pressure, NO concentration and NO output of the exhaled air. The NO concentration showed a stable plateau after an initial peak in measurements. For measurements of NO in nasally exhaled air, a tightly fitting mask covering the nose was mounted on the same antiviral filter as that used for oral exhalations, and the procedure was repeated likewise. All procedures were according to international recommendations (58, 59), but again, with the use of several flow-rates of air.

- Rectal NO (paper IV)

Rectal NO was measured using a previously described technique where an all-silicone catheter equipped with an inflatable balloon was inserted 10-12 cm into the rectum. The balloon was then inflated with NO-free air and incubated in the intestine for 10 minutes, allowing NO to diffuse into the balloon. The sample was aspirated and analysed immediately by a chemiluminescence NO analyser, where the peak NO concentration was registered (79).

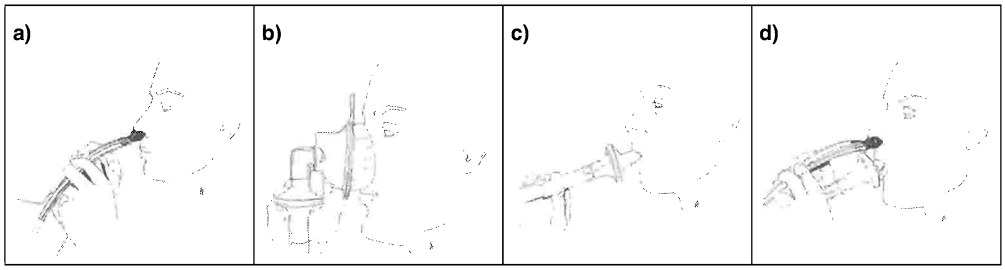

Figure 2. Different methods for the collection of airway gases. Nasal insufflation and the collection of air in a non-reactive bag during breath-hold (**a**), nasal exhalation (**b**), oral exhalation (**c**) and nasal aspiration or insufflation during oral exhalation against a resistance (**d**).

Study protocols

- CO in the upper airway (paper I)

A total of eighteen healthy non- or occasionally smoking volunteers participated in the study. In all subjects NO, CO and CO_2 levels were measured in nasally and orally exhaled, or nasally aspirated air. In addition, airway NO and CO was measured in the 7 occasionally smoking subjects 5 min after smoking a cigarette.

Following nasal inspiration to total lung capacity, nasal air was aspirated through a tightly fitting nasal olive at different flow-rates and led into non-reactive polyurethane bags impermeable to NO and CO. During sampling the subjects were asked to hold their breath with their mouth closed. NO-free and CO-free air was introduced into the other nostril through a second olive. In five of the subjects, CO-containing air was likewise introduced and contra-lateral CO was measured. For measurements on orally exhaled air the subjects were asked to inhale to total lung capacity from a clean air reservoir, then exhaling after a breath-hold, the last portion being collected in polyurethane bags.

- Methodological aspects (paper II)

Fifteen healthy subjects aged 20-30, seven women, were recruited for the study of nasally and orally exhaled NO. Measurements were performed repeatedly during one day, when the subjects were confined to the laboratory. Morning values were also measured on three different days.

Twenty-five healthy subjects aged 24-59, eight women, were recruited for the study on the effects of a mouthwash procedure. In order to minimise the contribution of NO formed non-enzymatically in the mouth to the levels of orally exhaled NO, the mouth was rinsed during one min using 10% sodium bicarbonate (18).

- Airway NO in inflammation (paper III-V)

HIV/AIDS (paper III): Thirty-one HIV patients aged 25-60, 22 men, were recruited from the outpatient ward of the Departement of Infectious Diseases at Huddinge University Hospital, Sweden. Twenty-six healthy controls were matched with regard to age, sex, height and weight. NO in nasally and orally exhaled air was measured, along with HIV-specific markers in blood. All NO measurements were performed before lunchtime, utilising the newly introduced single-breath nasal subtraction method (paper II).

Endotoxaemia (paper IV): The participants were ten men aged 21-43, with good health as confirmed by history, physical examination, electrocardiogram and haematological screening. After a 30-minute rest period, baseline samples were collected and endotoxin was given. The lipopolysaccharide (LPS) used was National Reference Endotoxin, E.Coli Lot.G 1, given in saline solution and administered intravenously over five minutes followed by a flush of saline. Blood samples for nitrite/nitrate were collected at baseline, four and six hours and for cGMP at baseline and four hours. Blood pressure, heart rate and temperature were registered hourly. Measurements of nasally and orally exhaled NO along with NO in rectal gas, sampling for blood gas content and haemoglobin were performed every hour. After four hours the subjects received 50 mg of the antiflogisticum diclofenac, and after 6 hours the experiment was terminated.

Allergic rhinitis (paper V): Eighteen patients aged 21-50, 9 men, and 18 healthy controls aged 20-46, 9 men, were included. The patients had ongoing, symptomatic and reportedly skin prick-positive non steroid-treated birch pollen AR, symptomatic strictly during pollen season and without diagnosis or symptoms of asthma. Seven patients who had previously experienced asthmatic reactions, which mainly had ceased during childhood or adolescence, were included. Patients who had occasionally taken intranasal steroids previous years refrained from doing so during the actual year of trial.

NO in nasally and orally exhaled air, and nasally aspirated NO, were measured at three different flow-rates of air, before and 30-50 min after intranasal administration of an NOS-inhibitor. The L-NAME (N^G-Nitro-L-arginine-methyl ester . HCl) was dissolved in phosphate buffer to a concentration of 100 mg/ml and final pH was 6.6. Five sprays of L-NAME (50 µl/spray) were administered into each nostril, resulting in a total of 50 mg. The mouth was first rinsed during 30 s using 10% sodium bicarbonate, and then for 15 s before repeated measurements. Scorings for allergic symptoms were also obtained from the patients, along with local pollen levels from the Swedish Palynological Laboratory at the Swedish Museum of Natural History.

Statistics

In study II, IV and V nonparametric statistics with two-sided p-values were used, based on the mean NO values at each time point. For the analysis of repeated measurements Friedman's test was used, and when significant followed by Wilcoxon signed rank sum test. For correlation analysis Spearman rank correlation was used. For unpaired data Mann-Whitney U test was used. For the analysis of paired data Wilcoxon signed rank sum test was used. A p-value of less than 0.05 was considered significant. Results are given as mean ± SEM or SD, as noted. In study III parametric statistics were used, including Student's t-test for comparison between groups and Pearson test for correlations.

RESULTS AND COMMENTS

CO in the upper airways (paper I)

The main and highly surprising finding of this paper is that we saw no evidence whatsoever for CO release in healthy human nasal airways, regardless of sample flow rate. In fact, a net resorption was found. This contrasts with a previous study addressing the issue of nasal CO release (78). This group later published a paper showing increased nasal CO levels in patients with seasonal allergic rhinitis, a study made using the same methodology as in the first paper (80).

Though our study found no CO, NO was present and its levels strongly flow-dependent in all subjects, as expected. Zero inlet levels of CO and NO were assured by the introduction of clean air through a tightly fitting olive inserted also in the contra-lateral nostril. Moreover, sampling was performed during breath-hold in order to avoid contamination from the lower airways. Furthermore, as expected, the levels of CO in orally exhaled air increased acutely after the subject had smoked a cigarette, showing that the highly sensitive method used indeed was capable of detecting differences in CO-output. Taken together, we believe these observations are sufficient to allow us to conclude that CO is not released from healthy human nasal airway. Also, our conclusion is supported by a study on allergic rhinitis, where no significant difference was found between nasally and orally exhaled CO in either patients or controls (77). Previous results on effects on NO of acute smoking in healthy non-habitual smokers have been inconclusive, and orally exhaled NO was unchanged in our study.

Our group has also published a study on lower airway CO release. If CO is released in the airway mucosa, one would expect a flow-dependency of the concentrations (81) in exhaled air. However, this was not the case, and it was suggested that the CO found in human airways is derived from the alveoli rather than the airway wall (82).

Methodological aspects of airway NO measurements (paper II)

This study shows that the NO levels in nasally and orally exhaled air of healthy human adults are clearly individual, with almost a 3-fold inter-individual difference in levels of NO in nasally exhaled air and a 10-fold inter-individual difference in levels of NO in orally exhaled air. This study also shows a rise in the NO levels in nasally and orally exhaled air over the course of the day, along with a relative consistency between days. We also found lower levels of orally exhaled NO in women, which were not related to body surface area. Furthermore, the mouthwash procedure did not influence levels of nasally exhaled NO, whereas it reduced both oral contribution to and methodological variation in the measurements of NO in orally exhaled air.

The inter-individual variation in airway NO output may indicate that it would be preferable for adult subjects to be their own controls in intervention studies, and the rise during the day may indicate that it would be preferable for subjects to be examined at the same time of day in future longitudinal studies, especially with a small number of participants or when only small differences in airway NO are expected. Also, these results would indicate that adult subjects and controls may have to be matched with regard to gender but not to height or weight in future case-control studies on exhaled NO, and when establishing normal values. The gender difference in orally exhaled NO has been confirmed in another study, also pointing out a need of adjustment for height and weight (83). However, in school children, it seems that normal

values should be related to age or bodyweight (84). In diseases with greater differences in values, such as in nasal NO output in patients with primary ciliary dysfunction (PCD), or when comparing larger groups, these considerations will probably not have to be taken into account.

Taken together, this study shows that 'nasal contribution to exhaled airway NO at a certain flow of air' can be calculated as the levels of NO in orally exhaled air after mouthwash subtracted from those in nasally exhaled air. Thus, this method for nasal NO measurements may be regarded as an alternative to the widely used method of nasal aspiration. One could argue that a nasal exhalation better reflects normal physiological conditions for trans-nasal air flow than the previously proposed aspiration/insufflation methods (58, 59). Also, the single-breath method is widely accepted for oral exhalations, and the nose mask was well tolerated and easily adapted to the set-up for oral measurements. This notion is supported in a study where the fixed flow exhalation appeared to be the preferred method, as it was highly reproducible and acceptable to the subjects (85).

Let us remind ourselves that this method optimally requires that there is no leakage round the nasal mask, and that oral cavity contribution to orally exhaled NO really is reduced to zero after mouthwash.

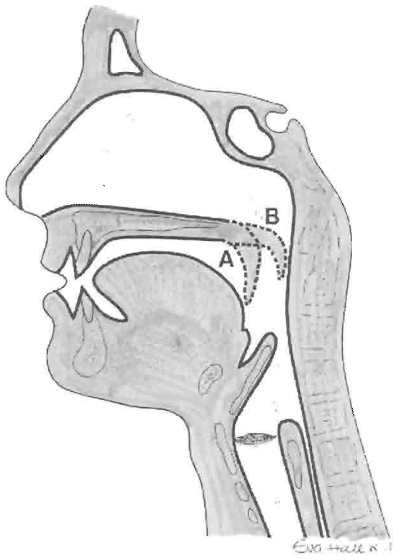

Figure 3. Contribution to nasally and orally exhaled NO from different compartments. The upper airway shown with the velum positioned as during nasal exhalation (A) or oral exhalation (B).

Nasally exhaled NO = supravelar space NO + $velum_A$ NO + lower $airway_A$ NO.
Orally exhaled NO = oral cavity NO + $velum_B$ NO + lower $airway_B$ NO.

If lower $airway_A$ NO ≈ lower $airway_B$ NO in the two instances,
and if $velum_A$ NO ≈ $velum_B$ NO,
and if oral cavity NO ≈ 0 after mouthwash,

then, supravelar space NO = nasally exhaled NO – orally exhaled NO after mouthwash.

Airway NO in inflammatory disorders (paper III-V)

The main findings of these three papers are that airway NO shows altered patterns in patients with systemic inflammation as well as in patients with inflammation localized in the airways.

- HIV/AIDS

We found that nasal NO release was reduced by about 20% in HIV patients compared to controls, whereas orally exhaled NO was similar. Correlation statistics in smoking HIV patients showed the number of cigarettes smoked per day to negatively correlate with orally exhaled NO, but not nasal NO.

We and others have earlier reported very low nasal NO in subjects with various airway disorders involving the sinuses, including PCD, CF and sinusitis. The mechanism underlying the reduced nasal NO in HIV patients can only be speculated upon at this stage. Perhaps the production of NO is down-regulated either as a direct consequence of the HIV infection or secondary to the infections caused by the immune deficiency. Alternatively, the passage and release of NO into the airspace of the nasal cavity is impeded in some way. Notably, patients with HIV have been shown to have mucociliary clearance delay (86) whereas patients with chronic sinusitis have normal ciliary beat rate in sinus biopsies (43). Among the various pathogens responsible for sinusitis in HIV patients are cytomegalovirus, mycobacteria and Aspergillus (87). Interestingly, these pathogens are all sensitive to NO in a number of experimental settings (88-90).

Nasal NO was not measured in a study previously undertaken on airway NO in HIV/AIDS, where reduced levels of orally exhaled NO were shown (91). Also, the measurement technique used in the cited study has been shown to mix air from the nasal passages into the orally exhaled air. In fact, it may have been that, even in that study, airway NO was reduced at the supravelar level. Alternatively, there may be differences in the studied patient populations that can explain the different results in the two studies.

Furthermore, habitual cigarette smoking is known to cause a reduction in orally exhaled NO. Nasal NO, however, did not correlate to smoking habits in the patients, suggesting that the reduced nasal NO in HIV patients was not caused by smoking.

- Endotoxaemia

The main finding of this study was that the levels of orally exhaled NO increased upon endotoxin administration, indicating enhanced production of NO from the lower airways or lungs. This orally exhaled NO increased in all subjects during the first two hours, reaching a plateau, then decreased back to baseline values one and two hours after administration of an antiflogistic. Also, plasma cGMP increased four hours after endotoxin. Increased heart rate, fever and the increase in systolic and decrease in diastolic blood pressure indicated that the volunteers responded to the endotoxin. However, nasally exhaled NO, rectal NO and nitrite/nitrate in plasma were not significantly changed.

Septic shock is a complex pathophysiological state, initiated by microbes or microbial products and characterized by systemic inflammation, circulatory insufficiency and altered substrate metabolism. Intravenous systemic administration of endotoxin to healthy volunteers produces changes in function similar to this, suggesting that endotoxin is a major mediator of the cardiovascular dysfunction in this condition (92). Endotoxin induces proinflammatory

cytokines such as TNF-α. Much of the inflammatory response induced by TNF-α depends on the activation of the transcription factor NF-κB. One of the many important genes activated in this way is iNOS. Excessive systemic NO production causes vascular dilation and systemic hypotension. NO also reacts with oxygen and superoxide to form reactive free radicals, which have numerous deleterious effects (93). Evidence for the role of overproduction of the vasodilator nitric oxide in sepsis syndrome has been presented (28). NO in luminal gases, as an inflammatory marker, from the upper airway, lower airway and gut has not been studied simultaneously in humans subjected to endotoxin infusion.

Exhaled NO has been extensively studied, mainly in patients with asthma. NO levels correspond well to the grade of inflammation and, inversely, to anti-inflammatory treatment with steroids. Further, NO in exhaled air has been shown to increase early after endotoxin challenge in animal models. In the present study we used the standardized method for measurement of exhaled NO recommended by the ERS and the ATS (58, 59). This includes e.g. exhalation against a resistance at a fixed flow-rate and allows for good separation of lower airway NO from nasal NO.

We believe that the early increase in NO production after endotoxin seen in our study indicates an augmented activity of cNOS in the lungs, since iNOS induction upon endotoxin and/or cytokines in the experimental setting is known to take at least 6-12 hours. Further studies will reveal if this method has a place in the ICU monitoring arsenal, for the purpose of rapidly and continuously measuring airway inflammatory response early in the course of sepsis.

- Allergic rhinitis

Somewhat surprisingly, we found that orally exhaled NO but not nasal NO was increased in patients with allergic rhinitis during pollen season. Notably, none had any symptoms suggestive of asthma. The decrease in nasal NO following L-NAME was greater in patients compared to controls in all modalities, being particularly pronounced at the highest flow rate used. Also, patients with the highest basal nasal or oral NO levels showed the largest decrease in NO levels. Nasal NO did not correlate with symptom score before or after L-NAME. There was no difference in either oral or nasal NO between patients who had previously experienced asthmatic reactions and those who had not. Nor was there any correlation between nasal NO and symptom score. The inter-individual spread in nasal NO was greater in the patients than in the controls, a finding that has been confirmed in another study (94). One way of interpreting this is that NO production in the nasal mucosa is generally increased in patients with allergic rhinitis, but at the same time, passage of NO from for example the sinus mucosa to the nasal cavity is impaired in some patients. All together, nasal NO levels seem to be determined not only by induction of NO synthesis in the inflamed nasal mucosa but also by the passage of NO from a production site distant from the nasal cavity where it is sampled. This fact may explain the varying results obtained in different studies. One general problem with nasal NO measurements in AR is the high background levels of NO from constitutive sources in the nose and sinuses. A small increases in nasal NO output may therefore easily be blunted. In this study we used several sampling techniques for estimation of nasal NO; therefore, it is unlikely that the lack of difference is explained by the method.

Despite the rather high dose of L-NAME used, the decrease in nasal NO was only 26-37% at 3 l/min. This is similar to what has been observed in earlier studies using similar doses. As proposed earlier this may be explained by minimal penetration of topically administered L-NAME into certain NO-producing compartments of the nose, e.g. the sinuses. Indeed, when high doses of NOS inhibitors are given i.v., nasal NO decreases approximately 65% (95), and

when given directly into the sinuses, sinus NO decreased by 80% (33). These findings support the notion that the paranasal sinuses may be major contributors to airway NO in healthy humans. Alternatively, the dose of L-NAME given was too low to produce a maximum inhibition of NO release.

Interestingly, mean oral NO was much higher in the AR patients, despite the absence of symptoms suggestive of asthma. This phenomenon may reflect an ongoing sub-clinical inflammation also in the lower airways, in atopic individuals with AR. It is a well-known fact that allergic rhinitis is a major risk factor for asthma. Whether high oral NO in AR patients indicates a greater risk of developing asthma later in life has yet to be proven.

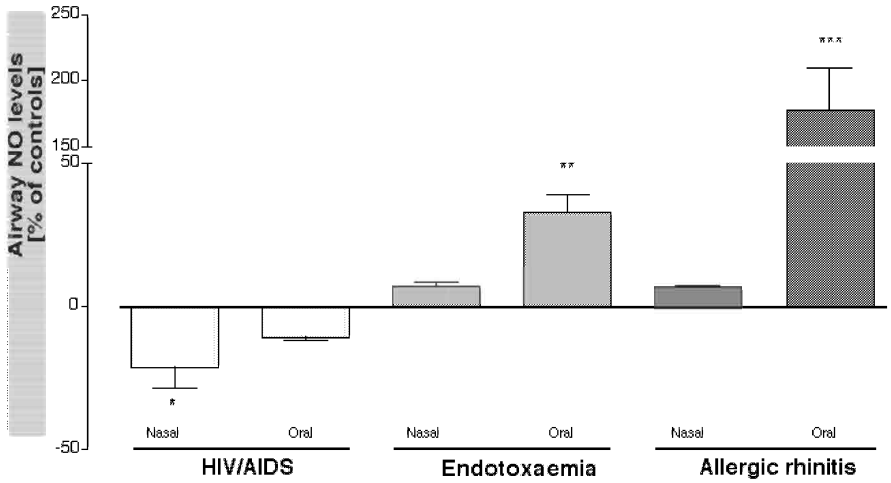

Figure 4. Change in nasal NO or orally exhaled NO compared to control (%) in HIV/AIDS, endotoxaemia and allergic rhinitis. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

GENERAL DISCUSSION

Nasal airway NO – What are we measuring?

The human nasal airway is indeed a complex system of canals and cavities, generating turbulent flow and protecting the lower airway by regulating temperature and humidity and by cleansing inhaled ambient air. Also, the nasal airway undoubtedly constitutes an important scource of the pluripotent gas nitric oxide (26, 32, 37). We may easily and non-invasively measure nasal NO. The NO measured will have come from different compartments in the nasal region. The levels found will depend on e.g. the method used, flow-rate of air, turbulence (paper V) and humming (96). However, we may not be able to discern precisely where the measured nasal NO comes from.

For the measurement of NO in air derived from lower airway, highly standardised procedures have been agreed upon. Orally exhaled NO is considered to reflect eosinophil (56), iNOS-mediated (49) airway inflammatory NO release (26, 50). However, this may not be the case in neutrophil-rich or non-atopic airway inflammation (97-99). In fact, measurement of exhaled NO was recently (2003) approved by the Food and Drug Administration in the US, and is on the way into the clinic as a means of non-invasively monitoring airway inflammation in asthma – a valuable complement to the methods currently in use.

Why should we measure nasal NO?

- To further elucidate the physiological role of nasal NO

Indeed, very high concentrations of NO have been found in the paranasal sinuses (32). This NO is believed to be involved in functions as disparate as primary host defence, including killing of microbes and stimulation of ciliary motility, inflammation and aiding oxygen uptake in the lower airway (42). However, the exact mechanisms by which NO is released and acts in the airway are far from settled. Because nasal NO measurements are so easy to perform, they will be important when further elucidating the physiological role of nasal NO in health and disease.

Disease	Exhaled NO	Nasal NO	Reference
Asthma	Increased	Unchanged	(26, 50)
COPD			
-stable	Unchanged		(100)
-unstable	Increased		(101)
Pulmonary hypertension/ Cor pulmonale	Reduced		(102)
Preeclampsia	Unchanged	Unchanged	(103)
Bronchiectasis	Increased		(104)
Fibrosing alveolitis	Increased		(105)
Primary ciliary dyskinesia/ Kartagener's syndrome	Very low	Very low	(45, 106-110)
Cystic fibrosis	Decreased	Decreased	(108, 111)
	Unchanged	Decreased	(53, 112, 113)
Diffuse panbronchiolitis		Very low	(114)
Symptomatic laboratory animal allergy	Increased	Increased	(115)
Nasal polyposis	Increased	Decreased	(94, 116)
-nonallergic		Decreased	(109)
Acute sinusitis		Decreased	(71)
Sinusitis in sepsis		Decreased	(117)
Chronic sinusitis		Unchanged	(109)
		Decreased	(72)
	Increased		(118)
Common cold		Unchanged	(71, 119)
	Increased		(120)
Experimental viral infection	Unchanged	Unchanged	(121)
	Increased	Unchanged	(122)
Wegener's granulomatosis			(70)
-active	Unchanged	Reduced	
-remission	Unchanged	Unchanged	
HIV	Reduced		(91)
	Unchanged	Reduced	(123)
Endotoxaemia	Increased		(60, 124)
Allergic rhinitis		Increased	(62)
	Increased	Unchanged	(53, 125)
	Increased		(126)

Table 1. Exhaled and nasal NO levels in different disorders involving the airways.

- Diagnosing and monitoring diseases (Table 1)

The genetic disease primary ciliary dyskinesia (PCD) often presents in the neonatal period. Diagnosis may require nasal biopsy under anaesthesia, and is often delayed. However, early diagnosis may prevent the development of bronchiectasis and the subsequent decline in lung function (127, 128). Measurement of airway NO is non-invasive, easily performed and yields instant results. Moreover, patients with PCD were found to have extremely low values of nasal NO, and also orally exhaled NO (45, 53, 106). As of recently, nasal NO measurements have been implemented as a screening tool for this disease, at national centres for PCD in the UK and the Netherlands (110, 128-130). This is indeed a rapid development - from discovery in 1994 to clinical implementation.

Cystic fibrosis (CF) is one of the most prevalent fatal hereditary diseases in caucasians, and chronic progressive lung disease is the major cause of morbidity and mortality. Even though sweat test is readily available in most hospitals, 10-15 % of patients with CF are not diagnosed until adulthood (128). Upon clinical suspicion, a sweat test can be performed after 2 weeks of age and in infants who weigh more than 3 kg, provided they fulfil certain criteria

concerning hydration, systemic illness and therapy (131). Interestingly, CF is also associated with considerably reduced nasal NO levels (53, 112, 113). This may mean that measuring airway NO will offer a non-invasive means for neonatal screening of CF. In fact, airway NO exhaled into a face mask has been found to be reduced in infants with CF (132). In patients where clinical suspicion of CF has been raised, it may also be important to consider alternative diagnoses, such as PCD (see above) or immunodeficiency (133).

Diffuse panbronchiolitis (DPB) is a chronic pulmonary disease, largely confined to individuals of Japanese, Korean or Chinese descent, and onset is usually in the second to fifth decade. As in PCD and CF, the common manifestation of chronic airway infection in DPB is attributed to abnormal mucociliary function. Chronic inflammation in the respiratory bronchioles leads to progressive obstructive respiratory dysfunction, cor pumonale and ultimately death, if left untreated. However, low-dose erythromycin has proven to be highly efficacious (134, 135). Interestingly, it has recently been found that nasal NO levels are greatly reduced in patients with DPB, making DPB the third known pulmonary disease with that characteristic. The report also stated that nasal NO measurements, which are non-invasive and easy to perform, may be a useful clinical test in these patients (114).

Interesting results have also been obtained concerning airway NO in nasal polyposis, with a negative correlation between nasal NO levels and polyp size scoring, notably with a concomitant increase in orally exhaled NO indicating inflammation in the lower airway (94, 116). Futhermore, it seems that in patients with nasal polyposis obstructing the paranasal sinus ostiae, the levels of nasally exhaled NO do not increase following humming, as do in healthy controls (96, 136, 137). Perhaps these observations of a refined methodology for nasal NO measurements, using humming, will lead to improvements in monitoring and therapy of patients with nasal polyposis.

The patency of the paranasal sinus ostia plays an important role in the pathophysiology of sinusitis. Blockage can alter the ventilation and drainage, leading to a change of the environment for microorganisms in the sinuses (138). Interestingly, hypoxia depresses nasal NO output (139), and it may be that this will occur in an occluded sinus. In fact, reduced NO levels have been found in the sinuses of septic patients with sinusitis (117), and in nasal air of children with acute sinusitis (71). Ostial blockage is an important factor contributing also to the chronicity of sinusitis (140). However, chronic sinusitis (CS) may be several entities. This may explain why one study has shown a decrease of nasal NO in conjunction with CS (72), whereas another has failed to do so (109). CS may be due to an immune inflammatory disorder, an infectious disease, or fungal allergy. The form of CS most typically associated with asthma is termed chronic hyperplastic eosinophil sinusitis (CHS) (141). Interestingly, a significant positive correlation was found between radiological signs of sinonasal inflammation, eosinophils in blood and sputum, and levels of exhaled NO in patients with CHS (118). So it may be that measuring both upper and lower airway NO will turn out to be useful in monitoring CS.

In patients with allergic rhinitis though, smaller differences in nasal NO levels, if any, have been shown compared to control (paper V). Measurements of nasal NO therefore do not seem particularly useful for these patients at present. In fact, it may be argued that an increase in nasal mucosa NO output cannot really be expected to show in nasal air. An increase of the same magnitude as that in asthma, will easily be blunted due to the high constitutive levels in the nasal airway (paper V). So it seems the methods need to be improved, in order to discern smaller differences in nasal NO output. Furthermore, we cannot really tell what the measured nasal NO reflects on the mucosal level, or what it says about symptoms in a patient. For example, we and others have found considerable inter-individual variation in nasal NO output

(study II), with an even greater range in patients with allergic rhinitis (paper V) (94). But no correlation was found between nasal NO levels and symptom scoring in the AR patients in our study. Also, in another study, no correlations were found between nasal nitrite and nitrate levels, nasal gaseous NO levels and symptom-scoring in allergic rhinitis (142). Accordingly, we did not find any correlation in the studied HIV patients between nasal NO levels and how many upper respiratory tract infections the patients had been through during the six months preceding the study (paper III). However, ostial blockage may also result from allergic rhinitis. And in our study, the inter-individual spread in nasal NO was increased in patients with AR. If it were found that patients with e.g. decreased nasal NO have occluded sinus ostiae, perhaps this may prove to be a way of identifying those at risk for developing sinusitis. The elevated levels of orally exhaled NO found in AR indicates an ongoing lower airway inflammation. Whether this, or a decrease in nasal NO, indicates a greater risk of developing asthma is yet to be determined.

It is well known that rhinitis, sinusitis, polyposis and asthma coexist. However, a causal relationship and a possible mechanism of action are still up for discussion (140, 141, 143-145). From the perspective of an otorhinolaryngologist, though, it is interesting to note that the non-invasive method of making nasal NO measurements before and after the subject has been humming, may offer a way to evaluate paranasal sinus ostial patency (96, 136, 137).

- Monitoring pharmacological manipulations of NO synthesis

The field of NO therapeutics is entering a crucial and exciting stage. Some patients may benefit from NO donating drugs, while in other cases inhibition of NO synthesis may be preferred (106, 108). Selective inhibitors of iNOS have been identified and will come to enter clinical trials. Reducing what is believed to be harmful overproduction of NO may have a therapeutic potential. However, inhibition of iNOS might be expected to increase susceptibility to infections and inhibit wound healing. Conversely, selective inhibition of nNOS might alter gastrointestinal and genitourinary function and cause spasm of sphincters. Inhibiting eNOS may lead to hypertension, enhanced white cell and platelet activation and increased atherogenesis. So, partial inhibition and/or tissue or cell-specific inhibition might be the ultimate goals (13, 146).

Regarding iNOS inhibition, one means of monitoring desired level of inhibition could be to measure nasal NO, since the major production site of upper airway NO in health seems to be a constitutively expressed iNOS-like enzyme in the paranasal sinus mucosa. Indeed, systemic inhibition of NOS through the use of the unspecific inhibitor L-NMMA has been shown to reduce nasal NO output by 50-65% (95, 147).

How should we measure nasal NO?

Obviously, nasal NO is easy to measure. And patients with some diseases may benefit from measurements, as discussed above. Several methods for nasal NO measurement have been suggested (study II) (58, 59, 85). However, different patients in different settings seem to require alternative methods.

The human nasal airway does not lend itself easily to being described in a compartment model to study the release of endogenously produced NO and the role it plays. However, some observations concerning nasal airway NO release seem to hold true – as the findings

have been repeated in several studies. For instance: very high levels of NO are found in the paranasal sinuses (32, 148), and nasal NO output is flow-dependent (study V) (64, 85, 149).

Consensus about measurement techniques has been achieved only to a degree. It is generally agreed that a standardized air flow of 3 l/min should be used, and that proper closure of the soft palate may be desireable. This closure can be obtained either through breath-hold, or through simultaneous oral exhalation against a resistance (58, 59, 150).

The method of nasal aspiration or insufflation by use of a trans-nasal air flow, requires tightly fitting olives (that may clog), air-pumps and a co-operative patient, because of the need for closure of the soft palate. The air will flow in both directions through the nasal cavity, entering and leaving the nose through nasal olives or some other device. This method may give us a value for NO release into the supravelar space at a certain flow of air (58, 59).

The single breath subtraction method (study II) requires an active patient, a tightly fitting nose mask and an efficient mouthwash procedure. It is easily adapted to the highly standardized method of measuring orally exhaled NO, according to the ATS (59). The air will flow uni-directionally through the nasal cavity, and may thus better reflect the normal physiology of breathing. Also, the nose and nostrils will be left unaffected. This method may also give us a value of supravelar space contribution to airway NO release at a certain flow of air.

Based on the results from study V, we have now performed further calculations, showing a significant correlation between the method of nasal aspiration and the single breath subtraction method at 3 l/min, both in controls and patients with allergic rhinitis (Fig. 5).

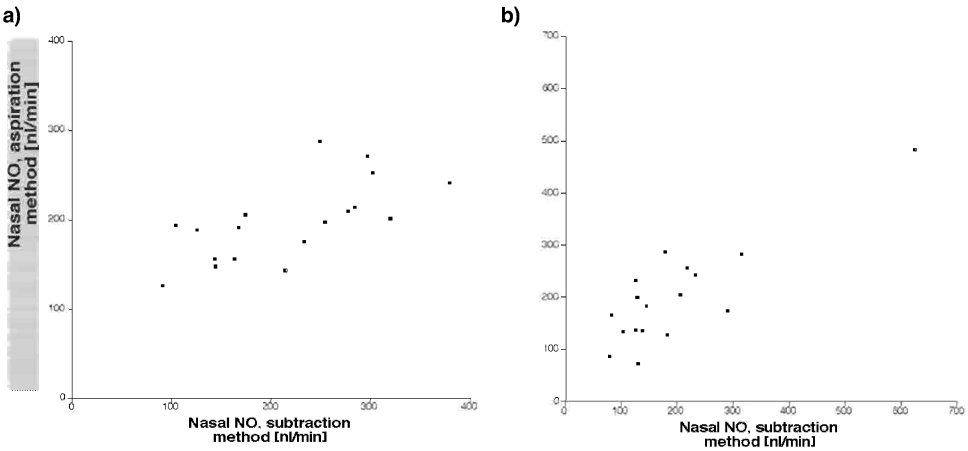

Figure 5. Correlation between nasal NO output at air flow rate 3 l/min, using the single breath subtraction method and the method of nasal aspiration in controls (**a**) and patients with allergic rhinitis (one outlier excluded) (**b**). Spearman r=0.7, p<0.001 and Spearman r=0.6, p=0.006. (Paper V)

These two methods are on-line measurements, i.e. requiring analysis on location. Collection of gases in e.g. non-reactive bags allows for off-line and multiple measurements to be undertaken (study I). If a small container is used (e.g. a syringe), air can be collected in an instant, and this method may give us a value of actual NO concentration in the nasal cavity.

In infants, a face mask can be fitted and air sampled by a mild aspiration, with or without closed nostrils, collecting the combined or upper or lower airway contribution to NO in breath (132, 151).

As mentioned, humming has recently been shown to greatly increase nasal NO, most likely by releasing the high concentrations of paranasal sinus NO into the nasal airway. This conclusion was based on the observation that the effect of humming was abolished in patients with nasal polyposis or chronic sinusitis (96, 136, 137).

Taken together, it seems that different methods for nasal nitric oxide measurements may be suitable in specific situations, and may be further improved in order to better be able to discern smaller differences in NO output. However, in diseases such as PCD, which causes NO levels to diverge widely from those seen in healthy subjects, methodology seems to be of minor importance.

Factor	Nasal NO	Orally exhaled NO	Paper
Acute smoking	No change	No change	I
Variation			II
- Inter-individual	Minor	Major	
- Over the day	Minor	Minor	
- Day to day	Minor	Minor	
Sex difference	No	Male>female	II
HIV/AIDS	Reduced	No change	III
-smoking, habit	No change	Reduced	
Endotoxaemia	No change	Increased	IV
Allergic rhinitis	No difference	Increased	V

Table 2. Change in nasal NO, nasally or orally exhaled NO due to various factors investigated in this thesis.

SUMMARY AND CONCLUSIONS

In summary, this study shows that,

Nitric oxide, but not carbon monoxide, is continuously released in the human nasal airways. (Paper I)

The NO levels in the airway of healthy humans are clearly individual, with almost a 3-fold inter-individual difference in levels of nasally exhaled NO, and a 10-fold inter-individual difference in levels of orally exhaled NO. Both nasally and orally exhaled NO show a rise over the course of the day. Levels of orally exhaled NO are higher in males than in females. These observations may have to be taken into account in future studies on airway NO release, and when establishing normal values.

The mouthwash procedure, aiming at increasing oral cavity pH, reduces both oral cavity contribution to, and methodological variation in orally exhaled NO. Taking these facts together, we suggest that nasal NO, or rather 'the supravelar space contribution to airway NO release at a certain flow-rate of air', may be calculated as the level of NO in nasally exhaled air minus the level of NO in orally exhaled air after mouthwash. (Paper II)

Nasal nitric oxide is reduced in patients with HIV/AIDS. Low nasal NO has been reported earlier in patients susceptible to respiratory tract infections, and a role for NO in primary host defence has been indicated. We speculate that the reduction in nasal NO, among other factors, may be involved in the decreased resistance to airway infections in these patients. (Paper III)

Orally exhaled NO increases for two hours following endotoxin infusion in healthy volunteers. Increase in heart rate, fever and systolic blood pressure indicates that the subjects indeed respond to the endotoxin. Further studies will reveal whether exhaled NO might be a valuable marker of sepsis-induced lung injury, and if monitoring of treatment is possible. (Paper IV)

Orally exhaled NO is increased, and the inter-individual variation in nasal NO levels is greater in patients with pollen allergic rhinitis during season. Further studies will reveal whether the patients with allergic rhinitis and decreased nasal NO are at particular risk of developing paranasal sinus disease, and if those with increased orally exhaled NO are at risk of developing asthma.

The decrease following intranasal administration of the NOS inhibitor L-NAME is greater in allergic rhinitis patients, in both nasal NO and orally exhaled NO, with the greatest difference found in nasal NO at the highest air flow used. We conclude that the methods for nasal NO measurements need to be further improved in order to detect smaller differences between health and disease. Possibly, an approach like this could be of use. (Paper V)

To conclude,

Nitric oxide is highly present in the upper airway of healthy humans, albeit with a substantial inter-individual variation. We have introduced a new method for nasal NO measurements, based on a highly standardized single-breath technique, giving information on the supravelar space contribution to airway NO release. This is an alternative to the method of nasal aspiration or insufflation.

In the diseases studied in this thesis, the influence of inflammation was examined by measurements of upper airway along with lower airway NO. We have found reduced nasal NO in patients with HIV/AIDS, increased orally exhaled NO in endotoxaemia (as a human model for sepsis), and high inter-individual variation in nasal NO along with increased orally exhaled NO in patients with allergic rhinitis.

Furthermore, there are airway inflammatory diseases in which nasal NO is extremely reduced, such as PCD, CF and DPB. Since nasal NO measurements are non-invasive and easy to perform, they may be useful for the screening, diagnosis and or monitoring of treatment of these diseases.

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