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HUMMING, NITRIC OXIDE AND PARANASAL SINUS VENTILATION

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To my family

ABSTRACT

The paranasal sinuses are air-filled bony cavities surrounding the nose. They communicate with the nose via the sinus ostia through which fluid and gases pass in both directions. A proper ventilation is crucial for sinus integrity and blockage of the ostia is a major risk factor for development of sinusitis.

In this thesis we have explored an entirely new approach to monitor sinus ventilation - the nasal humming test. We show in human studies *in vivo* and in a sinus/nasal model that the oscillating airflow generated during humming produce a dramatic increase in sinus ventilation. Interestingly, this increased gas exchange can be readily monitored on-line by simultaneously measuring the levels of the gas nitric oxide (NO) in nasally exhaled air. The sinuses constitute a major natural reservoir of NO and when gas-exchange increases during humming NO escapes rapidly into the nasal cavity thereby creating a highly reproducible peak in exhaled NO.
When exploring the different factors that determine the humming peak in NO we found that sinus ostium size was the most important but the humming frequency also influenced the sinus NO release. In patients with severe nasal polyposis and completely blocked sinus ostia the humming peak in NO was abolished. Moreover, in patients with allergic rhinitis, absence of a NO peak was associated with endoscopic signs suggestive of ostial obstruction. In the last study we went on to study if an oscillating airflow could be used not only to wash a gas out from the sinuses but also to enhance passage of substances into the sinuses. Indeed, we found evidence of an increased intra-sinus drug deposition by adding a sounding airflow to an aerosol.

In conclusion, the ventilation of the paranasal sinuses increases greatly when a person is humming; a finding that could have both diagnostic and therapeutic implications. Measurements of nasal NO during humming may represent a test of sinus ostial function. In addition, aerosol in combination with a sounding airflow could possibly be useful to increase the delivery of drugs into the paranasal sinuses.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals:

I. M. Maniscalco, E. Weitzberg, J. Sundberg, M. Sofia, J.O. Lundberg.

Assessment of nasal and sinus nitric oxide output using single-breath humming exhalations.

Eur Respir J 2003; 22: 323-9

II. J.O. Lundberg, M. Maniscalco, M. Sofia, L. Lundblad, E. Weitzberg.

Humming, nitric oxide, and paranasal sinus obstruction.

JAMA 2003; 289:302-3

III. M. Maniscalco, M. Sofia, E. Weitzberg, G. de Laurentiis, A. Stanziola, V. Rossillo, J.O. Lundberg.

Humming-induced release of nasal nitric oxide for assessment of sinus obstruction in allergic rhinitis: pilot study.

Eur J Clin Invest 2004; 34: 555-560

IV. M. Maniscalco, M. Sofia, E. Weitzberg, L. Carratù, J.O. Lundberg.

Nasal nitric oxide measurements before and after repeated humming maneuvers.

Eur J Clin Invest 2003; 33: 1090-94

V. M. Maniscalco, M. Sofia, E. Weitzberg, J.O. Lundberg.

A sounding airflow enhances aerosol delivery into the paranasal sinuses.

Accepted for publication in Eur J Clin Invest

CONTENTS

LIST OF ABBREVIATIONS

AP	allergic nasal polyposis
AR	allergic rhinitis
CRS	chronic rhino-sinusitis
cGMP	cyclic guanosine monophosphate
cNOS	constitutive nitric oxide synthase
eNOS	endothelial nitric oxide synthase (NOS-1)
F_ENO_{50}	fraction of exhaled nitric oxide
HS	healthy subjects
iNOS	inducible nitric oxide synthase (NOS-2)
L-NAME	N^G-nitro-L-arginine methylester
NAR	Nasal airway resistance
nNOS	neuronal nitric oxide synthase (NOS-3)
NO	nitric oxide
NO_2	nitrogen dioxide
NO_2^-	nitrite
NO_3^-	nitrate
PCD	primary ciliary dyskinesia
SD	Standard deviation
SEM	standard error of the mean

INTRODUCTION

The paranasal sinuses are hollow, air-filled cavities located in the skull bone of many mammals including humans. Their physiological function remains completely unknown but millions of people are painfully reminded of their existence each year during episodes of sinusitis.

The sinuses communicate with the nasal cavity through bony channels and this connection is crucial for sinus integrity[1]. A proper ventilation of the sinuses ensures entry of fresh oxygen and removal of waste products and invading bacteria. During a common cold or allergic rhinitis, swelling of the nasal mucosa may obstruct the sinus ostiae, an event considered to be central in the pathogenesis of sinusitis. Despite the connection with the nasal mucosa, which is heavily colonized by bacteria, healthy sinuses are considered to be sterile. One explanation for this could be the constantly ongoing local generation of nitric oxide (NO), a gas with antibacterial and ciliary stimulating properties. Parts of the NO gas generated inside the sinuses will leak out into the nose where it can be measured with non-invasive techniques.

Humming is identified in this thesis as a simple manoeuvre to greatly enhance the ventilation of the paranasal sinuses. By using nasal NO measurements we here further explore the physiological determinants of humming-induced sinus ventilation as well as potential diagnostic and therapeutic possibilities.

ANATOMY AND PHYSIOLOGY OF THE PARANASAL SINUSES

The biological function of the paranasal sinuses is still an enigma, although several theories have been put forward. These include humidification and warming of inspired air, lightening of the skull, improvement of vocal resonance, absorption of shock to the face or skull, and secretion of mucus to assist with air filtration[2-5].

The four paranasal sinuses (maxillary, sphenoid, frontal and ethmoid) develop as outpouchings of the nasal mucosa. At birth they are fluid filled and pneumatization occurs gradually during childhood so that by the age of 12 years all sinuses are fully developed. They remain connected to the nasal cavity via narrow ostia with a lumen diameter of 1 to 3 mm (Figure 1). The sinuses are lined with a mucosal membrane, which is thinner and less richly supplied with blood vessels and glands than the mucosa of the nasal cavity. The ostia of the frontal, maxillary and anterior ethmoid sinuses open into the osteomeatal complex, which lies in the middle meatus lateral to the middle turbinate. The posterior ethmoid and sphenoid sinuses open into the superior meatus and sphenoethmoid recess. The osteomeatal complex is important because the frontal, ethmoid and maxillary sinuses all drain through

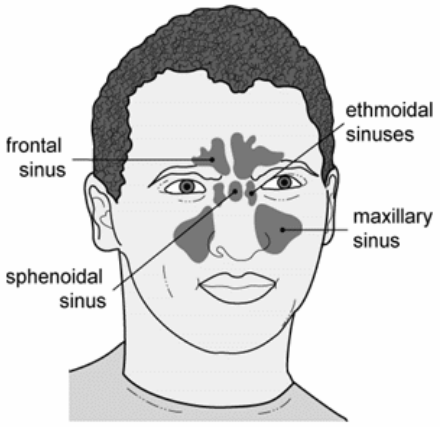

Fig. 1. The paranasal sinuses

this area. An effective ciliary transport system sweeps mucus towards the ostia which helps to to keep the sinuses free of invading pathogens[6]. In addition, a variety of antibacterial compounds are released from the sinus mucosa including lactoferrin, lysozyme and secretory antibodies[7]. For a satisfactory function of these protective mechanisms, the mucosa is dependent on adequate ventilation and blood flow. A normal ostial patency is the main prerequisite for adequate ventilation of the sinuses[5], and indeed an impaired ostial function is a central event in the pathophysiology of sinusitis[8].

NITRIC OXIDE IN THE UPPER AIRWAYS

Until less than a couple of decades ago the gas NO was considered to be only a noxious component of air pollution present in car exhaust and cigarette smoke[9, 10]. Although the discovery of an endogenously produced endothelium-derived relaxant of arterial vessels dates back to 1980[11], only in 1987 this molecule was identified as NO by two independent groups[12, 13]. Since then this research field has been continuously growing, and NO is now regarded as a key regulatory factor implicated in the control of several different physiological and pathophysiological processes including blood flow, platelet aggregation, neurotransmission, immunity[14] and inflammation[15].

In 1991 Gustafsson and co-workers[16] discovered that NO is present in exhaled breath of humans and two years later Alving and colleagues[17] could show that exhaled NO is increased in patients with asthma. These findings triggered a great interest in studying various aspects of exhaled NO and as of today more than 1000 papers have been published in this area.

Before I continue with a detailed description of the specific projects included in this thesis I would like to give a short background of NO in the airways including also a general overview of NO's chemistry, physiology and pathophysiology.

NO Chemistry and Synthesis

NO is a colourless gas characterized by a complex chemistry[18]. It is a small lipophilic molecule, which diffuses readily through over biological membranes. In normal conditions only a small fraction remains in aqueous solution, while the vast majority enters the gas phase[19]. Because of its chemical structure, characterized by the presence of an unpaired electron (making it a free radical), NO reacts rapidly with a number of biological compounds which helps to explain its diverse biological effects[14].

NO is synthesized from the semi-essential amino-acid L-arginine and molecular oxygen, in a reaction catalyzed by one of several forms of nitric oxide synthases (NOS). To date, three isoforms of NOS have been identified. Two of these are constitutively expressed, while the third is expressed only in activated cells. One form was initially identified in neurons of both brain and enteric nervous system, thus being termed neuronal NOS (nNOS)[20], whereas the other was originally found in endothelial cells lining the vasculature and therefore termed eNOS[21]. These two forms have been fully characterized and also found to be distributed more widely than originally thought, thus being renamed NOS-1 and NOS-3, respectively. The third isoform, originally named iNOS and now also known as NOS-2, is not expressed in resting cells

but is activated in response to various inflammatory stimuli such as bacterial products and cytokines.[18,22] NOS-1 and NOS-3 are calcium-calmodulin dependent and are activated in response to a calcium signal. Enzyme activation occurs rapidly and transiently providing a rapid pulse-like NO release. NOS-2 is calcium-calmodulin independent and NO synthesis is controlled at the transcriptional level. Once the enzyme is expressed it will produce large amounts of NO for prolonged periods. NOS-2 expression is dependent on transcription factors such as nuclear factor-κB[23] and activated by pro-inflammatory cytokines including tumor necrosis factor-α and interleukin-1β[24].

Soon after the L-arginine/NO pathway was described, several NOS inhibitors were developed and these have been useful tools in exploring the various physiological effects of NO. The majority of these compounds are L-arginine analogues with no selectivity for the different NOS isoforms[25, 26]. Anti-inflammatory glucocorticosteroids have been shown to inhibit NOS-2 expression[27] while leaving the constitutive enzymes unaffected.

Sources of Airway NO

After the discovery of exhaled NO in 1991, it was surprisingly shown that the upper airway is the major source of NO in adult healthy subjects[17]. Measurements in tracheostomized individuals clearly demonstrated that more than 90 % of all exhaled[28] NO originates from the nasal region[29]. The exact origin of the NO found in nasal air, as well as the relative contribution from different sources within the nasal airways are still debated. The paranasal sinuses are a particularly important source of NO, as shown by Lundberg *et al.*[30], who punctured maxillary sinuses and detected a continuous NO synthesis yielding very high concentrations (3000-25000 ppb). Interestingly, the NOS found in the sinuses is predominantly calcium independent[31], a characteristic usually attributed to the NOS-2, but studies showed that it is constitutively expressed and not inhibited by steroids, the latter being typical features of constitutive NOS. Immuno-histochemical and mRNA *in situ* hybridization experiments confirmed a high expression of NOS-2 in healthy sinus epithelium while this enzyme was much less expressed in the nasal mucosa. All together these findings led to the suggestion that much of the NO measured in the nasal cavity is originating from the paranasal sinuses.

On the other hand, other studies suggested only a partial contribution from the sinuses. Thus, in one healthy volunteer, the occlusion of both osteomeatal complex and spheno-ethmoidal recess, decreased nasal NO concentrations by only 12%, thereby suggesting only a limited role of paranasal sinuses as sources of nasal NO[32].

All three NOS isoforms have been identified in the upper airways. Several cells in the upper airways can express NOS-1 and 3 including parasympathetic neurons innervating nasal vessels, endothelial cells, and ciliated epithelial cells[33]. NOS-2 has been detected in epithelial cells, macrophages, neutrophils, endothelial and vascular smooth muscle cells.[33-35]

Physiological Role of NO in the airways

It has been suggested that NO contributes to non-specific host defences against bacterial, viral and fungal infections. Indeed, some bacteria are sensitive to the action

of NO at concentrations of only 100-1000 ppb[36]. Therefore, NO may help to maintain a sterile microenvironment within the paranasal sinuses, where the concentration of this gas can exceed 30 000 ppb [30]. NO could also contribute to the airway host- defence by regulating ciliary motility[37]. Indeed, it has been shown that application of NO donors to the human nasal mucosa stimulates ciliary beating[38], and that low nasal NO levels are associated with an impaired mucociliary function[39]. Furthermore, the NO substrate L-arginine is able to increase ciliary beat frequency *in vitro*[10, 40].

A role of NO has been hypothesized in the regulation of nasal airway resistance to airflow (NAR), and in the mechanisms of humidification and warming of inhaled air flowing through the nose. As a powerful vasodilator, NO could control the filling of nasal capacitance vessels, thus contributing to the total resistance to nasal airflow. Vascular tone also regulates nasal temperature, and variations of NO levels are indeed associated with thermic changes of nasal air in humans. Holden *et al*.[41] observed that inhibition of NO release is associated with cooling of nasal air. These findings suggest a potential role for NO in modulation of the vascular changes necessary for temperature conditioning of nasal air and, as a consequence, NO could also be involved in thermoregulation. However, in normal conditions NO does not seem to play an important role in regulation of vascular tone. In fact, Silkoff *et al*.[42] reported that local application of NOS inhibitors does not modify nasal patency measured by acoustic rhinomanometry. Accordingly, no effect on NAR was demonstrated after topical administration of L-NAME to healthy subjects, at doses capable of significantly reducing nasal NO levels[43].

NO has also been thought to exert other important physiological effects, such as the improvement of ventilation–perfusion ratio in the lungs after auto-inhalation[44, 34, 45]. In this sense, NO is believed to be an "aerocrine messenger" between the upper and lower airways.

NO and Upper Airway Diseases

Allergic Rhinitis

The nasal mucosa of rhinitic patients is characterized by a high expression of the inducible isoform of NOS-2[34] similarly to what is found in the lower airways of asthmatics[46-49]. In these subjects, NOS has been detected in the mucosal epithelium as well as in glandular cells and in the endothelium of sinusoids[50]. Kang *et al.*[51] suggested a role for NOS-2-derived NO in the increased mucus secretion occurring in rhinitis. It is also possible that NO plays a role in the control of NAR, and in the mechanisms of plasmatic microvascular leakage induced by pro-inflammatory substances in both healthy individuals and rhinitic patients subjected to allergen challenges[43, 52, 53].

An intricate picture arises from the studies assessing nasal NO levels in allergic rhinitics, where the results are quite contradictory. Some authors have shown variable nasal NO levels after exposure to allergens[54-57]. In these patients, an increased mucosal expression of NOS-2[34] was found as well as an increase in NO metabolites (nitrate and nitrite) in nasal lavage fluid[58]. Martin *et al.*[57] detected high nasal and oral NO in allergic rhinitic patients. Kharitonov *et al.*[56] reported that during the pollen season nasal NO, but not oral NO concentrations, were significantly increased in subjects with seasonal allergic rhinitis. These authors also showed that one hour after

nasal challenge with allergens, nasal NO decreased and this correlated with the severity of rhinitic symptoms. In contrast, Palm *et al.*[59] revealed no change in nasal NO levels in patients with allergic rhinitis, whereas in that study orally exhaled NO was increased. Also other groups failed to find an evident increase in nasal NO in seasonal rhinitis out of the pollen season[60] and during the pollen season[55].

Nasal polyposis

A high NOS-2 expression and activity has been demonstrated in nasal polyps[50]. Parikh *et al.* found that NOS activity was also increased in nasal polyps from aspirin-sensitive asthmatics[61]. They formulated the hypothesis that the overproduction of cytokines occurring in aspirin-sensitive patients stimulates NOS-2 expression, thus promoting an elevated synthesis of NO. The latter might inhibit the apoptosis of eosinophils, leading to an increased survival of these cells, which in turn would release a great amount of cytokines responsible for the perpetuation and propagation of nasal inflammation.

Studies have shown that nasal NO levels are lower in patients with polyps when compared to controls or individuals with uncomplicated allergic rhinitis[62]. These results, which are apparently paradoxical with regard to the already mentioned high levels of NOS-2 expression, were attributed to the blockage of the osteomeatal complex and to the consequent inability, for newly generated NO, to reach the nasal cavity. The authors of this study suggested that nasal NO synthesis depends on iNOS expressed by the inflamed nasal mucosa, as well as on constitutive sinus production by normal tissues.

Sinusitis

In both acute and chronic sinusitis, a reduction of nasal NO levels and NOS-2 expression has been reported[63, 64]. In children with acute sinusitis, Baraldi *et al.* demonstrated that nasal NO concentrations increased when clinical conditions improved as a consequence of antibiotic therapy[64]. It is still uncertain whether the low NO levels detected in acute and chronic sinusitis result from a reduced maxillary NO production, or are rather mainly due to an obstruction of sinus ostia caused by local oedema, nasal congestion, and mucus accumulation within the sinus cavities. Indeed, the nasal mucosa swelling which develops during rhinitis might also lead to a partial blockage of sinus ostia, thus resulting in a reduced passage of NO from sinuses to the nasal cavity, where it is measured. The latter interpretation is corroborated by the results of studies performed on patients with nasal polyposis, showing that nasally measured NO concentrations were inversely correlated with the number of occluded paranasal sinuses. However, the hypothesis of a reduced maxillary NO synthesis is supported by the finding of strongly reduced NOS-2 expression in the sinus mucosa during sinusitis.

Primary Ciliary Dyskinesia (PCD)

PCD is a genetic disorder characterized by abnormal structure and/or function of ciliated cells in the airways and elsewhere[65]. Clinical presentations include neonatal respiratory distress, recurrent lower respiratory tract infections, chronic rhino-sinusitis and male infertility. It is associated with visceral mirror image arrangement (situs inversus) in 50% of cases. PCD should be diagnostically distinguished from bronchiectasis, atypical asthma, cystic fibrosis and unusually severe upper airway disease[66]. Diagnosis is difficult and often requires complex investigations, aimed to

evaluate the beating frequency of cilia, their light microscopy morphologic pattern as well as ciliary ultrastructure and orientation, assessed by electron microscopy[66]. In 1994 Lundberg *et al.* reported markedly reduced nasal NO levels in 4 children with PCD[28], a finding that has now been confirmed by other groups[67, 68]. Consistently, an almost absence of nasal NO has been noticed, thus suggesting that NO measurement may be used as a screening test for PCD, with a high degree of diagnostic accuracy.

NO Measurement Methods

General considerations of measurement technique

The most common way of measuring exhaled and nasal NO is by chemiluminescence[69], where detection depends on the photochemical reaction between gaseous NO and ozone generated in the analyzer. In brief, NO contained in the sampled air reacts with an excess of ozone 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.

Other techniques that have been used to establish that NO is present in the exhaled breath of humans include mass spectroscopy and gas chromatography-mass spectroscopy.[16, 29]

Methods for nasal NO measurement

The development of standardized and reproducible methods for measurement of nasal NO has come on the heels of methods developed for measurements of exhaled NO. While the American Thoracic Society (ATS) and European Respiratory Society have agreed on a highly standardized procedure for measurements of lower respiratory tract exhaled NO, it has not been possible to define one single standardized measurement procedure for nasal NO measurements[69, 70]. Nasal NO measurement techniques have been addressed by a European taskforce, and more recently by an official statement of the ATS, reflecting the growing interest in these measurements[71].

The simplest and most used method for measuring nasal NO has been to aspirate nasal air directly from the nasal cavity using the intrinsic flow of the NO analyser[59, 72-74]. Typically, the sampling probe is connected to a nasal olive, which is gently introduced into one nostril. Then the subject is asked to hold his/her breath and air circulates from one naris to the other around the posterior nasal septum.

Another method to measure nasal NO involves a nasal single-breath exhalation at a fixed flow rate using a face-mask[75, 76]. In this case an oral NO exhalation is also performed using the same exhalation flow and the value is subtracted from that obtained during the nasal exhalation[77].
The sampling technique sometimes needs to be adjusted depending on the situation and the patients to be studied. For example, breath-holding or single-breath measurements are not possible in non-cooperating individuals e.g. infants and sedated patients.
With all methods, a constant trans-nasal flow rate produces a washout phase followed by the establishment of a steady NO plateau seen in the profile of NO versus time,

analogous to that seen in the lower respiratory tract. Nasal NO concentration is inversely related to the trans-nasal airflow rate. However, different flow rates may have different aerodynamic profiles resulting in changes in the physics of airflow (e.g. laminar versus turbulent flow)[78]. The aerodynamics of this flow may affect the nasal NO output. For all the above reasons, any standardized method used should rigorously control trans-nasal airflow rate. The product of trans-nasal flow rate and measured NO concentration allows calculation of NO output. Present evidence suggests that NO is relatively constant over a range of trans-nasal flow rates between 0.25-3 liters/minute[78]. There is reasonable agreement, using different measurement techniques, that nasal NO output is in the range of 200-450 nL/min in healthy primates. At higher flow rates, NO may increase progressively[77].

It is becoming clear that, due to the complexity of anatomical structures, measurements of nasal NO output or concentration cannot provide evidence as to the source of the gas (e.g. nasal cavity and/or paranasal sinuses) or the biochemical processes, which generate the NO. The sinuses communicate with the nasal cavity through ostia, and there is a continuous gas exchange between these cavities. Therefore, a high NO flux from sinuses could easily blunt slight alteration of NO originating from nose. This may explain the large intra and inter-individual variation in nasal NO has been reported both in healthy people and in subjects with rhinitis.

At present there is no simple and non invasive tool to explore the great NO reserve contained in the sinuses and the exchange occurring between sinuses and nose via sinus ostia. In 2002 Lundberg and Weitzberg[79] reported that nasal humming (which is the production of a tone without opening the lips or forming words) resulted in an enormous increase in exhaled nasal NO levels, likely due to a rapid washout of sinus NO caused by the oscillating air flow. This thesis is a direct continuation of that finding and here we tried to explore if measurements of NO during humming could be used to overcome some of the problems associated with the currently used methods.

AIMS

The overall aim of this thesis was to investigate the diagnostic and therapeutical potential of nasal humming. The specific aims were:

- To explore the various factors influencing the nasal NO increase induced by humming.

- To study nasal NO during humming in patients with nasal and/or sinus diseases.

- To assess if humming could be used to improve the reproducibility of nasal NO measurements.

- To test if a sounding airflow could be used to enhance the penetration of a drug into the sinuses.

MATERIAL AND METHODS

For a detailed account of materials and methods the reader is referred to the individual papers.

STUDY SUBJECTS

All studies were approved by the local ethics committees and all subjects gave their informed consent.

In **paper I, II, IV** and **V**, a total of 38 healthy non-smoking volunteers (aged 20–47 years) without any history of allergy, nasal disease, asthma or any other chronic lung conditions were recruited from hospital personnel.

In **paper II** we studied 10 subjects with nasal polyposis and in **paper IV** 5 subjects with allergic rhinitis associated with nasal polyposis were studied. All patients had bilateral polyps and completely opaque sinuses according to a recent computed tomography scan and they were on the waiting list for sinus surgery.

In **paper III** and **IV,** 15 + 59 consecutive untreated subjects with mild to moderate allergic rhinitis were studied. All had positive skin prick testing for common allergens. They were referred from their general practitioner to the skin-testing facility because of a previous clinical history of nasal obstruction, rhinorrea, itching and sneezing, lasting ≤ 5 years and occurring at least for two consecutive months/years. They required medical treatment with nasal decongestants and/or antihistamines for less than one week per month and they had nasal symptoms not troublesome enough to interfere with normal daily activity or night time sleep.

SINUS/NASAL MODEL

In **paper I** and **V** a two-compartment model resembling the nasal cavity and one sinus was used (Figure 2).

Description of the model.
A syringe (representing the sinus) was filled with various NO gas concentrations between 2 – 10 ppm and connected horizontally to a plastic cylinder (representing the nasal cavity) *via* a luer fitting. The diameter of the syringe tip (representing the ostium) was varied between 0.8 – 4.0 mm. The volume of the syringe was varied between 5 – 20 mL. The distal end of the cylinder (nasal cavity) was left open or connected to a Hans Rudolph resistor of 50 cmH_2O/L s, thereby generating cylinder pressures of either 1 or 10 cmH_2O. Flow and pressure were measured by a linear pneumotachymeter. Resulting NO output was measured at the distal end of the cylinder by a rapid-response chemiluminescence system (Aerocrine AB, Stockholm, Sweden). The signal output from these devices were connected to a computer-based system (Aerocrine NO system; Aerocrine AB) and yielded an instant on-screen display of flow, pressure, NO concentration and NO output.

Artificial generation of humming in the model.
Pressurised NO-free air was set to generate three different flow rates (0.20, 0.25 and 0.30 L/s). The air was led through the plastic cylinder (nasal cavity) either *via* a

rubber duck call, which yielded a pulsating airflow, or *via* a rubber duck call without the sound generating membrane (quiet control). Three duck calls with different fundamental frequencies (120, 200 and 450 Hz) were used. NO was measured during a 10-s period and all experiments were repeated five times. In an additional experiment, a turbulent flow was generated by leading pressurised NO-free air through a plastic mesh connected to the cylinder and NO was measured as described above. This experiment was carried out without a sound-generating device.

Human humming in the model.
In the same model, the pulsating airflow was also generated by a subject performing oral exhalation through the cylinder, with or without phonation, at two fixed flow rates (0.20 or 0.25 L/s) and three different frequencies (130, 150 or 450 Hz). NO output was calculated from the entire exhalation with subtraction of oral NO output. All experiments were repeated five times. To estimate the rate of NO exchange between the two cavities, the remaining NO concentration in the syringe at the end of each experiment was also measured.

Measurement of artificial and human humming sound frequency.
The audio signal of humming was picked up by a TCM 110 Tiepin electret condenser microphone placed on the plastic cylinder in the model (Figure 2) and recorded directly onto a PC by the Soundswell Signal Workstation. The fundamental frequency was extracted by its Corr module, which computes the autocorrelation of the audio signal in two adjacent time windows. The mean fundamental frequency and SD were then determined by means of its histogram module. The resonance frequency of the model system was calculated according to Durrant and Lovrinic.

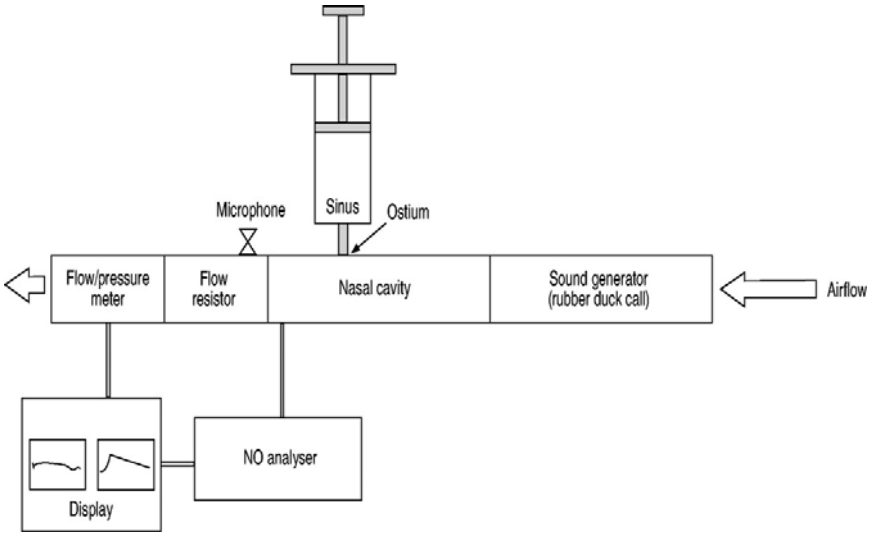

Figure 2. Schematic presentation of a model resembling the sinus (syringe), the ostium (syringe tip) and the nasal cavity (plastic cylinder). NO: nitric oxide.

MEASUREMENT OF EXHALED NO

The exhaled NO measurements were performed using chemiluminescence technique according to the ATS guidelines (**papers I - V**). NO levels were measured in oral and nasal exhalations either with phonation (humming) or during quiet expiration. A tight fitting mask covering the nose was used for nasal measurements, and a mouthpiece was used for oral exhalations. The subjects performed a single-breath exhalation against an

expiratory resistance at a fixed flow rate (0.2 L/s) for ten seconds. To calculate nasal NO levels the value of oral NO was subtracted from the nasally exhaled NO.
The exhaled air was led into the Aerocrine NO System or Niox® (Aerocrine AB, Solna, Sweden) or 280 NOA (Sievers Instruments, Boulder, Sensor Medics, Milan, Italy) for measurement of NO. Ambient NO levels were below 10 ppb during the measurements.

NASAL ENDOSCOPY

In **paper III** nasal endoscopy was performed with a rigid endoscope 2.7 mm 0°. For each side of the nose endoscopic images were evaluated by the following scoring system. 0 = absence of significant nasal obstruction, 1 = turbinate hypertrophy or polyps with a free visible passage to the osteomeatal region, 2 = severe obstruction resulting from nasal polyposis or marked middle turbinate hypertrophy and no visible passage to the osteomeatal region. Using this system the maximum total score was 4.

CLINICAL QUESTIONNAIRE

In **paper III** a clinical questionnaire about the patients present symptoms was used. This included registration of the following symptoms: nasal obstruction, rhinorrea, sneezing, itching, headache/facial pain and was scored as follows: 0 = absence, 1 = very mild, 2 = mild, 3 = moderate, 4 = troublesome. Maximal score with this system was 20. Subjects with a total score equal or less than 2 were classified as asymptomatic at the time of the study.

AEROSOL SYSTEM

In **paper V** an aerosol system was used to administer aerosol to the healthy subjects. In brief a jet nebulizer (Nebula) was connected via a T valve to a rubber duck call, which yielded a sound with pulsating airflow or to a rubber duck-call without the sound generating membrane. The rubber duck call was joined to a nasal cannula, while a flow generator was connected to the opposite extremity of the T valve.

STATISTICS

The NO output (nL/min) was calculated for all sampling modalities in all papers. Nasal NO was calculated using a subtraction method, where NO in orally exhaled air is subtracted from that in nasally exhaled air. Results were presented as mean ± SEM in **paper I** and as mean ± SD in **paper II, III** and **IV**. In **paper III** the 99% confidence intervals (CI.) were calculated for silently exhaled nasal NO values and net humming NO values. For analysis of unpaired data in **papers I-IV** the Mann-Whitney U test was used. For paired data the Friedman's test was used in **paper I,** the Wilcoxon's test in **paper I, III** and **IV** and ANOVA for repeated measurements in **paper V**. Correlations were analysed by Spearman correlation test.
In **Paper IV** the coefficient of variation for the different breathing modes was calculated by dividing the within subject standard deviation by the mean. The limit of agreement was calculated according to Bland & Altman[80]. Analysis of variability before and after humming was performed using Friedman test.
A p value less than 0.05 was considered significant.

RESULTS AND COMMENTS

CHARACTERIZATION OF NASAL NO DURING HUMMING (PAPER I)

We have characterised nasal NO during humming and explored various factors that could determine gas exchange between the sinuses and the nasal cavity. Moreover, we have investigated if NO measurements during humming could give additional information about NO production at different sites in the upper airways. This was achieved by studying healthy volunteers, as well as creating a model of the sinus and the nose (Figure 2), in which the influence on NO release from the sinus could be investigated in relation to ostium size, humming frequency, sinus volume, sinus NO concentration, air flow and pressure.

In all subjects humming caused an increase in exhaled NO output compared to silent exhalation (Figure 3). In contrast, oral phonation had no effect on exhaled NO. Furthermore, in all the experiments the shape of the NO curve was characterized by a large initial peak followed by a progressive decline towards a plateau (Figure 3). Our interpretation of these findings is that humming induces a rapid release of air from the sinuses, containing high concentrations of NO, which is then followed by a plateau representing the combined continuous release of NO from the nasal and sinus epithelium. Topical nasal application of the NOS inhibitor L-NAME reduced silently exhaled nasal NO by > 50%, but had no effect on the humming-induced increase in nasal NO output.

When consecutive humming manoeuvres were performed at 5 sec. intervals nasal NO output decreased during each manoeuvre mainly because of the gradual disappearance of the initial peak. Within four nasal humming manoeuvres the peak was completely absent. A silent period of 3 minutes was necessary to obtain a complete recovery of the nasal NO output during humming. Nasal NO output during silent exhalation was reduced by 30 % when performed immediately after a humming procedure. These findings together with those from the L-NAME experiments point towards a substantial contribution of sinus NO to the levels found in nasally exhaled air. Furthermore, it suggests that humming procedures before nasal NO measurements may enhance this method when used in patients with nasal diseases. This is further explored in **paper IV**.

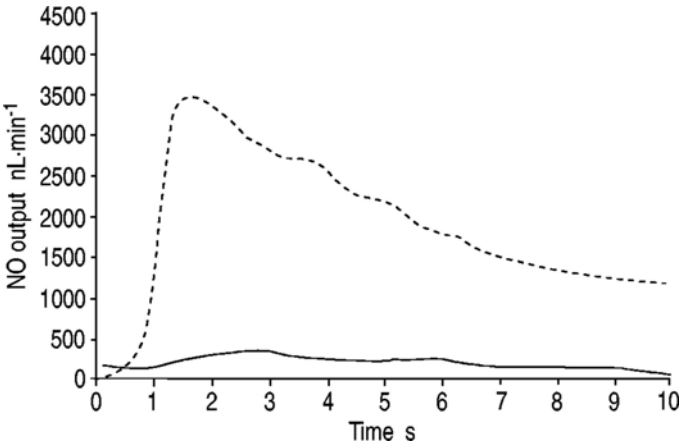

Fig. 3. Representative tracing of nitric oxide output (NO) during a single-breath nasal exhalation performed at constant flow with (dotted line) or without (solid line) humming.

In the model, all the factors tested, which are known to influence normal sinus ventilation had effect on NO output during humming. However, ostium size was the most critical, shown by a dramatic increase in NO output after enlarging the ostium size (Figure 4). In fact, with an ostium diameter above 2 mm more than 80% of the sinus gas volume was exchanged by one single humming manoeuvre. Also modification of humming frequency caused significant changes in NO output.

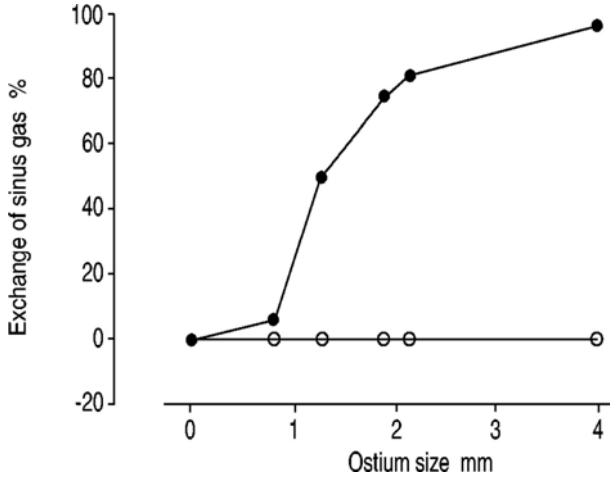

Fig. 4. Influence of ostium size on sinus gas exchange in a sinus/nasal model during silent exhalation (○) or humming (●).

When studying the effect of different humming frequencies on NO output in the model, we found the greatest effects where achieved when the humming frequency was close to the calculated resonance frequency of the particular sinus (Figure 5). Turbulent flow did not change NO output during humming. The results from the model indicate that the sinus ostium is the major determinant for NO release from the sinuses during humming.

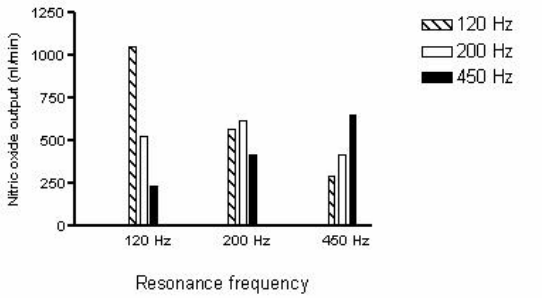

Fig. 5. Effect of humming frequency on nitric oxide output in a sinus/nasal model using sinuses with different resonance frequency.

HUMMING NO AND NASAL DISORDERS (PAPER II AND III)

Nasal polyps (II)

Based on the findings in **paper I** we hypothesized that patients with obstructed sinus ostia would exhibit less of an increase in exhaled nasal NO levels upon humming. Therefore we studied patients affected by bilateral nasal polyps, chronic sinusitis and completely opaque sinuses and compared the results to those obtained in healthy controls. NO levels were measured in single-breath nasal exhalations first silently and then during humming.

During silent nasal exhalation, NO output was similar in control patients and patients with sinusitis (189 ± 27 nL/min vs. 162 ± 22 nL/min, respectively). Mean output of nasal NO increased 7-fold during humming (to 1285 ± 189 nL/min) in control patients but remained completely unchanged in the patients with sinusitis (169 ± 21 nL/min, Figure 6). The most likely explanation is a lack of air passage between the sinuses and the nasal cavity, which was evident from the computed tomography showing obstructed sinuses bilaterally in all patients.

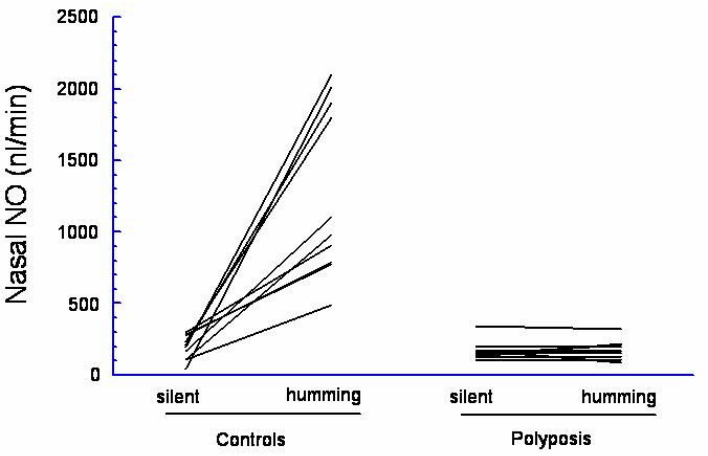

Fig. 6. Effect of humming on nasal nitric oxide output in healthy controls and in patients with bilateral nasal polyposis.

Interestingly, one of the patients had endoscopic sinus surgery during the course of this study, and in this patient NO levels increased during humming to almost normal levels 2 weeks after the operation (figure 7).

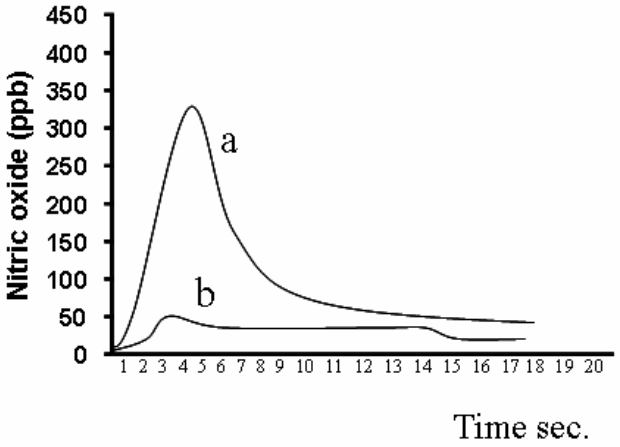

Fig. 7. Effect of humming on exhaled nasal NO concentrations in a patient with nasal polyposis before (b) and two weeks after surgery (a).

It is noteworthy that humming was necessary to reveal differences in nasal NO between patients and controls since silent nasal exhalation levels of NO were similar in these groups.

Allergic rhinitis (III)

Sinusitis and nasal polyposis are increasingly recognized as a common event occurring during upper allergic respiratory diseases such as allergic rhinitis. Indeed, as many as 45-60 % of patients with allergic rhinitis have radiological evidence of sinus abnormalities and allergic rhinitis is considered a major risk factor for sinusitis [81].

In the **paper III** we studied if nasal NO measurement during humming could be used to identify sinus obstruction in patients with allergic rhinitis. Nasal NO levels were measured during humming in rhinitis patients and the levels were compared to nasal symptoms and endoscopic findings.

Consecutive patients with mild to moderate persistent allergic rhinitis were studied and their present nasal symptoms were recorded. Then NO levels were measured during

silent single-breath nasal exhalations and humming. Based on the NO results the patients were divided in two groups; those with a great increase in nasal NO during humming (humming responders, n = 46) and those without a significant increase (humming non-responders, n = 13).

A significant humming response was then defined as a humming NO increase above 3 standard deviations from the mean NO levels measured during quiet nasal exhalation in the study population. Subjects who showed a humming NO level of at least 3 standard deviations above the mean NO levels during quiet exhalations were therefore categorized as humming responders while those who did not increase above this level were categorized as humming non-responders.

In 11 of the non-responders and in 22 responders, the passage to the osteomeatal complex area was assessed and scored by nasal endoscopy. This was done by an oto-rhino-laryngologist unaware of the NO results. Among the non-responders 9 out of 11 patients (80%) had endoscopic signs suggestive of bilateral sinus obstruction, compared to 1 out of 22 (< 5%) of the humming responders. Baseline nasal symptom score and NO levels during quiet exhalation were not significantly different between the groups.

In conclusion the absence of a nasal NO increase during humming is associated with endoscopic findings suggestive of sinus ostial obstruction in subjects with allergic rhinitis. Measurement of nasal NO during humming may be a simple method to detect sinus abnormalities in these patients.

REPRODUCIBILITY OF NO MEASUREMENTS IN RELATION TO HUMMING (PAPER IV)

Large intra and inter-individual variations in exhaled nasal NO have been reported both in healthy people and in subjects with rhinitis. We hypothesized that nasal NO measurements would have a better reproducibility if they were performed immediately after repeated humming procedures, since pre-treatment with humming would reduce the contribution from the paranasal sinuses in the following silent exhalations.

NO output was measured before and after repeated humming manoeuvres in orally and nasally exhaled air in healthy subjects (HS), patients with allergic rhinitis (AR) and patients with allergic nasal polyposis (AP). NO measurement were performed in each subject during silent nasal exhalations, either preceded by a period of silence/free speaking or immediately after five consecutive humming manoeuvres (post-humming).

Mean nasal NO output after a period of silence/free speaking was 231 nL/min (95% CI: 178 – 284) in HS, 434 nL/min (347 - 522) in AR ($p<0.001$) and 262 nL/min (163 - 361) in AP. After repeated humming manoeuvres nasal NO output was 16% (5 to 50%) lower in HS and 14 % (1 to 49%) lower in AR, while it remained unchanged in the subjects with AP. Intra-subject coefficient of variation during silent nasal exhalation was 12%, 13% and 5% in HS, AR and AP, respectively. After humming intra-individual coefficient of variation significantly decreased in both HS and AR, but was unchanged in AP (Figure 8).

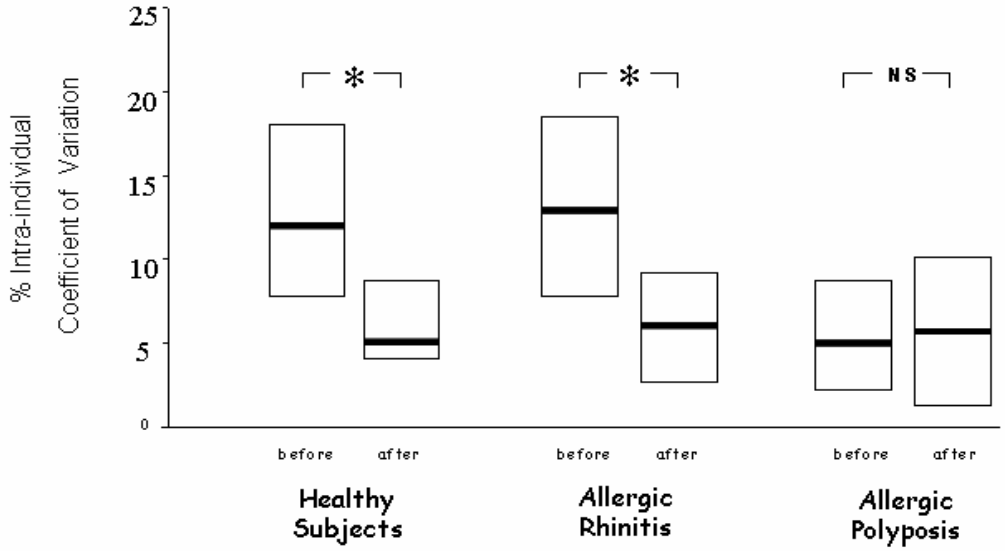

Fig. 8. Mean value (horizontal bar) and 95% confidence interval (box) of intra-subject coefficient of variation of nasal nitric oxide output measurements taken during standard quiet nasal exhalation before and after five consecutive humming manoeuvres. $^{*}P< 0{\cdot}05$. NS = not significant.

These data show that nasal NO levels measured immediately after repeated humming manoeuvres are consistently lower and more reproducible than nasal NO levels measured after a period of silence or free speaking. The humming procedure may be useful to better estimate NO output from the nasal cavity mucosa in health and disease.

SOUNDING AIRFLOWS AND AEROSOL DELIVERY INTO THE SINUSES (PAPER V)

The use of aerosol therapy in the treatment of paranasal disorders has been suggested by several authors. Administration with a nebulizer is not without difficulties and several factors such as particle size, aerosol pressure and other factors may limit the ability of proper delivery of solutions. Since an oscillating airflow improves the gas exchange in the paranasal sinuses we wanted to test if it could also enhance the deposition of an aerosol into these cavities.

In healthy subjects the NOS inhibitor L-NAME was administrated into the nostrils by a jet nebulizer connected to a duck pipe, which could be manipulated to generate either an oscillating or silent airflow. The degree of penetration of L-NAME into the sinuses was estimated by calculating the reduction in nasal NO during humming exhalations performed after nebulization.

Sinus drug deposition was also studied in a model of the nose and sinus, in which changes in sinus volume, ostium size and sound frequency was evaluated. The calculated resonance frequency of the model was 200 Hz.

In humans the nasal delivery of L-NAME with a silent airflow had no effect on the NO levels during humming (from 201.4 ± 75.3 ppb to 181.0 ± 82.3 ppb; p= 0.67). In contrast, the delivery of L-NAME with an oscillating airflow caused a significant reduction in nasal NO during humming (from 225.6 ± 61.5 ppb to 147.5 ± 40.1 ppb; $p<0.05$). The effect on humming-induced nasal NO release lasted at least 40 minutes (Figure 9). The two different modes of L-NAME delivery reduced silently exhaled NO from the nose to a similar degree. Control experiments with saline showed no effect on nasal NO levels. These findings suggest that nebulization with an oscillating airflow may enhance the delivery of a drug into the sinuses.

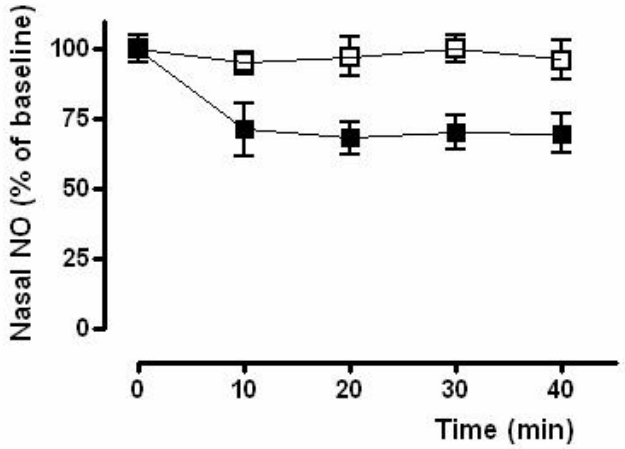

Fig. 9. Exhaled nasal nitric oxide measured during humming after topical aerosolized L-NAME delivered with (filled squares) or without (unfilled squared) an oscillating airflow.

In the model of the nose and sinus the aerosol delivery with an oscillating airflow caused a significant increase in the weight of the syringe (sinus) as compared to the delivery without an oscillating airflow (4.4 ± 0.3 mg and 1.0 ± 0.1 mg, respectively, $p < 0.05$). This further supports that oscillation improves sinus deposition during nebulization.

Ostial diameter influenced the weight of the syringe when the aerosol was delivered by an oscillating airflow. Furthermore, a significant change in aerosol deposition was observed by modifying the sound frequency. The maximum deposition was found at a frequency of 200 Hz as compared to 120 Hz and 450 Hz (4.4 ± 0.3 mg, 3.5 ± 0.2 mg and 3.0 ± 0.3 mg, respectively; $p < 0.05$) . No difference in aerosol deposition was noted when syringe volume was modified, regardless of mode of delivery.

GENERAL DISCUSSION

Measurements of exhaled NO are now becoming a part of clinical practice in the diagnosis and therapy monitoring of allergic asthma[82-86]. This simple non-invasive test reveals the degree of lower airway inflammation and can help the physician when titrating anti-inflammatory treatment, monitoring compliance or in differential diagnosis[87-89]. There has been hope that also nasal NO measurements could be similarly useful e.g. in monitoring allergic rhinitis but to date the results have been somewhat disappointing mainly due to methodological issues.

In this thesis we have explored an entirely new approach to nasal NO measurements - the nasal humming test. These studies hopefully open up new opportunities to use nasal NO measurements in diagnosis and therapy monitoring of upper airway inflammatory disease. In addition, therapeutic opportunities using humming can also be foreseen.

Below, our specific findings are discussed in greater detail. I have divided this discussion into three major areas: first a section on the mechanisms behind the increase in nasal NO during humming, thereafter a section on the possible diagnostic application of a humming test and finally a more speculative section on the possible therapeutical application of humming.

SOURCE OF THE NITRIC OXIDE MEASURED DURING HUMMING

The great peak in nasal NO is seen only during humming whereas a silent exhalation at the same flow rate does not produce any increase in nasal NO. So clearly in some way the sound waves are facilitating NO release from the airways. The source of this NO must be within the nasal airways as the NO peak only occurs during nasal exhalations with humming and not during oral exhalations. Where then is the NO coming from, and what is the mechanism of the release? In this thesis several proofs have been given to support that the nasal peak in NO during humming is a result of a rapid washout of NO from the sinuses. First, the profiles of the nasal exhalation curves (peak and progressive decline) in the model (**paper I**) and in the human in vivo studies (**paper I** to **IV**) were almost identical and showed characteristic patterns. In addition the NO release during humming was dependent on the same prime parameter (ostial size). A rapid increase in NO occurs when a subject starts humming, and after the initial maximum, NO starts to decrease. Naturally, in the model NO reaches zero when the syringe is emptied while in the *in vivo* studies this does not happen because of the continuously ongoing production of NO. Second, after removal of the accumulated NO from the paranasal sinuses during humming, the concentration is gradually rebuilt again. The longer time allowed between two humming manoeuvres, the higher the NO peak in nasal NO. Indeed, both the peak and the total nasal NO output were markedly decreased following repeated consecutive humming manoeuvres and a complete recovery was observed after a 3-min period of silence. This pattern fits well with the notion that humming rapidly empties the sinuses and that a period of silence will allow for NO to accumulate again. Third, topical nasal administration of a NOS inhibitor (L-NAME) did not affect nasal NO during humming, while it significantly decreases silent nasal NO. This suggests that the great NO amount released during humming is likely to come from the sinuses which were not targeted by the aerosol administration.

In recent studies other authors have made very similar observations on humming and nasal NO using slightly different methods[90, 91]. Again, a rapidly increasing NO

concentration was found when the subjects started humming. After reaching a maximum, the emission started to decrease with the shape of an exponential decay and finally reached a constant level. The time constant of this decay (NO washout) was 3 sec. The peak height of the NO emission during humming increases when the time between two humming processes increases. When no humming-induced NO emission takes place, the NO concentration in the sinuses re-build again.

HOW DOES HUMMING INCREASE SINUS VENTILATION?

In our study significant changes were found in NO output by modifying the frequency of humming. In particular when studying the effect of different humming frequencies in the nasal/sinus model, it was found that the NO output was greatest when the frequency of humming was close to the calculated resonance frequency of the particular sinus (Figure 5). The influence of frequency was also analyzed in human subjects by modulating the sound frequencies of humming and measuring NO output. The results confirm a dependence on the frequency that varied considerably between subjects.

It is likely that the sinus cavity behaves like a kind of Helmholtz resonator[92] , which is a container of gas with an open hole (neck or port). Such a container has a specific resonance frequency. At that frequency the air within the neck (airplug) will be most easily moved. An outside variation in air pressure causes the airplug in the neck to oscillate in and out. This oscillation is explained by the following: When air is pushed into a cavity through the neck (ostium) it will be compressed, its pressure will increase and it will tend to expand back to its original volume, thereby driving the air back out of the cavity. The momentum of this driving force will create an overshoot and produce a slight vacuum in the cavity which will then suck air back in. The air will oscillate in and out of the cavity for a few cycles at a natural frequency. It is comparable to a mass on a spring, which is pressed down and then released (Figure 10). During humming the oscillating sound will move the air in and out through the ostium, with the highest efficacy near the resonance frequency of the particular sinus. The vibration of the airplug will pump NO out of the cavity resulting in the observed peak in nasal NO seen during humming.

Several factors will determine the resonance frequency of the system including the area of the opening port, the cavity volume and the length of the opening port. Very recently, Granqvist *et al.*[93] developed a physical model where airflow from a pressure tank was modulated at different frequencies. This airstream was passed through a tube with a radial hole constituting the neck of a Helmholtz resonator which contained NO. The NO content of the air streaming out of the tube was measured. This NO content varied when the location of the resonator, its air volume or the modulation frequency of the airflow were changed.

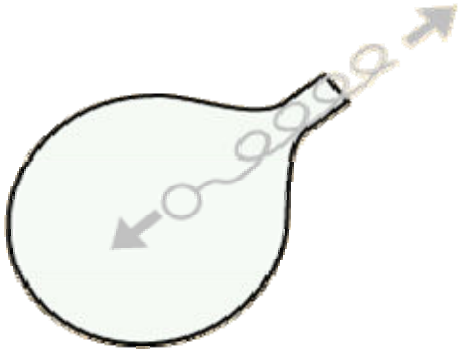

Fig. 10. During humming the sinus cavity behaves as a kind of Helmholtz resonator. The humming oscillations induce movement back and forth of the airplug in the neck of the container like a mass on a spring. This movement increases the gas exchange from the container.

The relevance of these three factors was also confirmed in a computer model of the system. The computer model was surprisingly efficient in replicating data obtained from the physical model of the nasal tract and maxillary sinuses.

It is clear from the present raw modelling of the nasal resonator system that the increased NO ventilation that occurs during humming can be explained by the acoustic flow in and out of the maxillary sinuses.

We cannot exclude that humming could increase NO output either from other sources in the main nasal airways or with other mechanisms such as turbulence of flow or vibrations of skull. However, oral NO output does not increase during humming indicating that pulsating sound waves do not increase NO diffusion from NO-producing respiratory epithelial surfaces in general. Furthermore the application of a turbulent flow in the model did not have any effect on NO.

FACTORS WHICH INFLUENCE NO LEVELS DURING HUMMING

In **paper I** we attempted to explore the factors that determine the rate of air exchange between the sinuses and the nose. For this we created a sinus/nasal model in which we could vary the studied parameters, e.g. ostium size, sinus volume, sinus NO concentration and humming frequency. We found that all these factors influenced the nasal NO release during the humming, but the ostium size was the most important. In fact when doubling the ostium size the increase in NO washout was almost 10 fold. This confirms earlier studies which have shown that ostium size is the most important factor determining sinus ventilation. Aust and Drettner[94] were the first to measure sinus ostial patency *in vivo* in man. They used an invasive technique to measure pressure differences in the sinuses after injecting air and then calculated the resistance to outflow via the natural sinus ostium[95]. More recently, Paulsson *et al.*[96] used a non-invasive 133-xenon washout technique to evaluate the ventilation of the sinuses in healthy subjects and in patients with sinus disease. The washout halftimes were used as a measure of the ventilation of the sinuses. They found that the ostial size was the most important factor determining the washout of 133-xenon from the sinuses[97]. In polyposis patients the washout times were significantly longer than in healthy subjects, and became significantly shorter after sinus surgery[96].

It should be noted that the model we used in our study does not mimic the continuous NO production occurring normally in the human sinuses and the complex dynamics of production and absorption from nasal airway mucosa. Moreover, the sinus ostium diameter could not be directly measured in the healthy subjects. Nevertheless, the experiments looking at remaining NO levels in the syringe after single-breath exhalations indicate that humming is an enormously effective means of increasing sinus ventilation. This is also supported by the *in vivo* experiments (**paper I**), where the rapid decline in NO during humming indicated sinus gas exchanging.

HUMMING IMPROVES ACCURACY OF NASAL NO MEASUREMENTS

Attempts to standardize nasal NO measurements have been recently made[70]. However, the possibility to detect increases in nasal NO output is limited by the high background NO levels in the upper airways originating from several sources in the nose and sinuses. As we have discussed above, a high NO flux from the sinuses could easily

blunt slight alterations in NO originating from nasal mucosa. Accordingly, large intra and inter-individual variation in nasal NO have been reported both in healthy people and in subjects with rhinitis[54-57]. Interestingly, by introducing repeated humming manoeuvres immediately before measurements the NO levels measured during silent exhalation were reduced as was the intra-individual variation (**paper IV**). This is likely explained by the rapid removal of NO accumulated in the sinuses thereby transiently reducing sinus contribution to NO levels measured in nasally exhaled air. This is interesting for several reasons. First, it gives support to the notion that sinus NO is indeed continuously contributing to the levels of nasal NO. Secondly, it indicates that if nasal NO is measured after repeated humming manoeuvres it may represent a more reliable measure of the nasal mucosal NO output with less contribution from the paranasal sinuses. Although we managed to decrease the variability in nasal NO by humming prior to the measurements, it remains to explore if this procedure will increase the diagnostic accuracy of nasal NO measurements.

A HUMMING TEST TO DIAGNOSE AND MONITOR SINUS DISEASE?

Acute or chronic inflammation of the paranasal sinuses and the adjacent nasal surface, clinically termed rhino-sinusitis[98] is very common. It has been reported that sinusitis has an incidence rate of 14% in USA, with an 18% increase over the past 11 years[99]. Moreover, sinusitis is increasingly recognized as a common event occurring during upper and lower allergic respiratory diseases such as allergic rhinitis and asthma[53, 100]. Indeed, as many as 45-60% of patients with allergic rhinitis have radiological evidence of sinus abnormalities and allergic rhinitis is considered a risk factor for sinusitis[81]. In clinical practice, classic symptoms of blocked nose, rhinorrea, sneezing and itching are commonly encountered either with rhinitis or sinusitis. This often requires further objective testing such as nasal endoscopy and radiological investigation, which are time and cost-consuming, thereby contributing to the high economic burden of allergic rhinitis[100, 101]. A key pathogenic event in sinusitis is the impaired sinus ventilation caused by obstruction of the osteo-meatal complex[101]. This area of the nose is difficult to assess during routine clinical examination[102].

We speculate that measures of nasal NO may represent a new approach to test osteomeatal patency. In **paper II** we found a complete absence of the normal nasal NO increase during humming in patients with bilateral nasal polyposis and CT-proven occluded sinus ostia. Furthermore in **paper III** the humming peak was lost or markedly reduced in allergic rhinitis patients with endoscopic signs suggestive of sinus obstruction. It is likely that the absence of a nasal NO peak during humming is caused by a partial or complete obstruction of the osteomeatal complex with reduced gas exchange through the sinus ostium. Also, from the experimental model of the nose/sinus in the **paper I**, the ostium size was shown to be the major factor determining the NO increase seen during humming.

Interestingly, in the prospective study (**paper III**) in allergic rhinitis subjects there were no significant differences in clinical nasal score between humming non-responders and humming responders whereas the endoscopic score was significantly higher in the first group. This indicates that a humming test could be used to reveal sub-clinical sinus obstruction. If a humming test accurately reflects the degree of osteal function, the next step would be to explore if this information has any clinical relevance. That is, will

early interventions (e.g. with anti-inflammatory treatment) in certain risk patients prevent later development of chronic sinus disease?

HUMMING TO TREAT RHINO-SINUSITIS?

As evident from this thesis, humming is an enormously effective means of increasing sinus ventilation. Previous works have shown that the time needed to exchange all gas in the sinuses varies between ~5 min up to 1 h[94, 96], with much longer time needed in patients with sinus disorders[96]. The current results indicate that almost the entire sinus volume is exchanged in one single exhalation if the subject is humming. Even when using a small ostial diameter, humming was very effective at increasing NO exchange in the sinus model. This suggests that humming could help to increase sinus ventilation in patients with sinusitis and partly obstructed ostia.

Whether this would be beneficial in treatment or prevention of sinusitis remains to be studied. Nevertheless, it is interesting that medical, as well as surgical treatment of chronic sinusitis generally aims to increase sinus ventilation, which is often impaired in this disorder.

In support of this, a case report was described very recently[103] proposing that strong low pitch (130 Hz) humming was an effective means of treating chronic rhino-sinusitis (CRS). In this report a subject hummed frequently for 4 days in an attempt to treat the sinusitis. The morning after the first humming session, the subject awoke with a clear nose and found himself breathing easily through his nose for the first time in over 1 month. By humming 60–120 times four times per day CRS symptoms were essentially eliminated in 4 days.

Although the concept of using humming to prevent or treat sinusitis humming appears attractive, evidence of its effectiveness is still lacking. A potential problem is in a situation with completely blocked ostia when sinus ventilation obviously will remain zero even during humming.

HUMMING TO ENHANCE DRUG PENETRATION INTO THE SINUSES?

The ability to deliver medications directly into sinuses could theoretically reduce the need for systemic medications thereby reducing the risk for unwanted side effects [104]. Several factors may influence how effectively a nebulizer will deliver solutions to the target organ including the particle size, aerosol pressure and other factors[105, 106]. In spite of the widespread use of aerosols in respiratory diseases[107-118] few studies have been performed in order to assess aerosol deposition into the sinuses cavities[119-122].

In **paper V** we have shown that the peak in nasal NO caused by humming is markedly reduced if an aerosolized NOS inhibitor is delivered into the nose by the use of a sounding airflow. In the control experiments, where the same NOS inhibitor was delivered without the sounding airflow, the humming-induced increase in nasal NO was unaltered. This suggests that the sounding airflow enhanced the penetration of the NOS inhibitor into the NO producing cells in the sinuses. Furthermore, the aerosol delivery with sounding airflow increased the aerosol deposition in a model of the paranasal sinus, where the size of ostium and frequency of the sounding airflow influenced the aerosol deposition.

To our knowledge this is the first study, which gives a demonstration of the effect of sound pulsated aerosol to enhance aerosol diffusion into sinuses both *in vivo* in humans and in a model of a sinus. Kumazawa *et al.*[109] found that intermittent vocalisation increased deposition rate of aerosol particles into the larynx of healthy subjects. Proof of this concept for nasal cavities has arisen from several studies mainly performed in artificial models and in animals or from deposition studies using radionuclide or more recently from a plastinated model of the maxillary sinus[123].

CONCLUSIONS

The overall conclusions of the present thesis are the following:

- Ventilation of the paranasal sinuses is greatly enhanced by humming
- The increased sinus ventilation is detected as a peak in exhaled nasal NO
- Ostium size is a major determinant of the NO peak seen during humming
- The humming peak NO is absent in patients with chronic sinusitis and osteomeatal obstruction
- Endoscopic signs of sinus abnormalities are associated with reduced NO peaks during humming in patients with allergic rhinitis
- Nasal NO measured immediately after repeated humming may represent a more reliable measure of the nasal mucosal NO because contribution from the sinus NO source is transiently minimized.
- Aerosol in combination with a sounding airflow may increase the delivery of a drug into the paranasal sinuses.

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