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Nitric oxide production in the sphenoidal sinus by the inducible and constitutive isozymes of nitric oxide synthase*

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SUMMARY

Objective: *To study the production of nitric oxide (NO), and the presence of different isoforms of the NO-synthesising enzyme, NO-synthase (NOS), in the paranasal sinus.*
Materials and Methods: *Ten patients, undergoing surgery for pituitary adenoma, were examined for the presence of NO gas in the sphenoidal and maxillary sinus. The distribution of different NOS isozymes in mucosal biopsies from sphenoid and maxillary sinus and ethmoidal cells was studied.*
Results: *The mean concentration of NO was 2575 ppb in the sphenoidal sinus and 6792 ppb in the maxillary sinus. Morphological analyses revealed intense NADPH-diaphorase staining throughout the epithelium. Immunoreactivity against NOS2 (inducible NOS) was observed in the apical cell layer but not of the basal layer. NOS1 (neuronal NOS)-immunoreactivity was mainly seen in the subapical part of the epithelium and NOS3 (endothelial NOS)-immunoreactivity was observed only in the most apical part of the epithelium.*
Conclusion: *NO concentration in the sphenoidal sinus is about the same as in the nasal cavity and approximately half of the concentration found in the maxillary sinus. All of the three main different isozymes of NOS can be demonstrated in the mucosa of the sphenoidal and maxillary sinus and ethmoidal cells, NOS2 being the most abundant isoform.*

Key words: mucosa, nitric oxide, nitric oxide synthase, paranasal sinus

INTRODUCTION

The presence of nitric oxide (NO) in human airways was first demonstrated in 1991 [1]. A few years later it was revealed that the major part of NO in exhaled air originated from the upper airways [2, 3]. Lundberg and co-workers demonstrated a high production of NO and presence of nitric oxide synthase (NOS) in the epithelium of the paranasal sinus, and postulated that this NO enters the nasal cavity through the sinus ostia and may be the main source of nasal NO [4, 5].

Nasal NO is measurable in the nose even in newborns [4], and has not been shown to differ between sexes. Nasal NO-production is lower in patients with nasal polyposis, and in acute or chronic sinusitis, probably due to a decrease of sinus leakage [6, 7, 8]. However, nasal NO-production is not altered in common colds [9]. NO has a toxic effect on several micro-organisms, and is considered to prevent local infections in the paranasal sinus and the nose [10]. Infections in the paranasal sinus do occur, the most common paranasal infection being the maxillary sinusitis. The sphenoidal sinus is only affected in 16% of all cases of acute sinusitis [11].

We hypothesised that the concentration of NO is higher in the sphenoidal sinus than in the maxillary sinus, explaining the low frequency of infections in the sphenoidal sinus.

Thus, we performed this study in order to compare the production of NO in sphenoidal sinus air to that of the maxillary sinus. Furthermore, we determined the distribution of the three different isoforms of the NO-producing enzyme, NOS, in the mucosa of the sphenoidal and maxillary sinuses and ethmoidal cells.

MATERIALS AND METHODS

Patients

Ten patients, who underwent surgery for pituitary adenomas via a lateral rhinotomy approach, participated in the study [12]. None of the patients had a tumour penetrating the sphenoidal sinus. The mean age was 55 years (15-82 years), and 7 were

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female. Four patients had treatment with peroral glucocorticoids, one was a smoker, and no one was treated with nitrates or NO-donors. All subjects received parenteral glucocorticoid (100 mg hydrocortison i.v.) prior to anesthesia (See Table 1). Patients were masked during induction of anesthesia and subsequently orally intubated. Approximately 30 minutes after intubation surgery was initiated and all gas samples and mucosal biopsies were collected during the first half-hour of surgery.
The project was approved by the Local Ethics Committee at Göteborg University, Sweden (S285-02). All patients gave their informed consent.

Table 1. Patient demography.

Pat no	Sex	Age	Hormone producing tumour	Smoker	Peroral gluco-corticoid treatment
1	F	57	No	Yes	No
2	F	51	No	No	No
3	F	82	No	No	No
4	M	52	Yes (GH) *	No	No
5	F	57	No	No	Yes
6	M	62	Yes (GH) *	No	No
7	F	15	No	No	Yes
8	F	48	No	No	No
9	M	58	No	No	Yes
10	F	67	No	No	Yes

* GH = growth hormone producing tumour

Measurements of NO in sinus and nasal air
All NO sampling was made by the same surgeon (BP). Nasal air was aspirated from the nasal cavity with a gastight 10 ml syringe during 30 seconds. A second air sample was taken after one minute. A lateral rhinotomy incision was made and the anterior bony wall of the left maxillar sinus was exposed [12]. A needle was pushed through the wall and an air sample was taken with a 5 ml gastight syringe, during 15 seconds. Thereafter the concha media was removed. The posterior septal wall was lateralized, and the bony anterior sphenoidal sinus wall exposed. A needle (connected to a thin plastic tube) was pushed through the wall, and 2 ml air was aspirated with a 5 ml gastight syringe during 15 seconds. All syringes were immediately sealed after air sampling and transported to the NO-analyzer. NO values remained stable in the syringe for > 2 hours at different concentrations of NO in the air.
Gas from the sphenoidal sinus was mixed with air to a volume of 5 ml, which is the smallest volume that can be used for the technical set apparatus. The gas was then injected into the chemiluminescence NO-analyzer (Modified Seres NOX 4000, Seres, Aix-en-Provence, France). The detection limit for NO was 1 ppb and calibrations were performed with known concentrations of NO in N2 (AGA, Stockholm, Sweden).

Histochemistry
Mucosal biopsies (2 x 2 mm) were collected from the sphenoidal sinus, the ethmoidal cells and the maxillary sinus. Tissue was fixed for at least 4 h in a 4°C solution of 4% formaldehyde in phosphate-buffered saline PBS; pH 7.4, and rinsed in 15% sucrose in PBS. The tissue pieces were then frozen at – 40°C in an isopentane solution and stored at –70°C until sectioning.
NOS immunohistochemistry. Cryostat sections were cut at a thickness of 10 µm and thaw-mounted onto chrome-alum coated slides as described previously [13]. The sections were incubated overnight with primary antisera against NOS1 (neuronal NOS; 1:2000; raised in sheep; Dr. P. Emson, Cambridge, UK), NOS2 (inducible NOS; 1:500; raised in rabbit; C19; Santa Cruz Biotechnologies, Santa Cruz, CA) and NOS3 (endothelial NOS; 1:500; raised in rabbit; C20; Santa Cruz Biotechnologies).
After the incubation period the sections were rinsed in PBS, and then incubated for 90 min with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit, 1:80 or 1:160 in PBS (Sigma, St. Louis, MO). Subsequently, the sections were mounted in glycerol/PBS with p-phenylenediamine to prevent fluorescence fading. In control experiments, no immunoreactivity could be detected in sections incubated in the absence of primary antiserum. The stained structures are referred to as NOS1-, NOS2-, and NOS3-immunoreactive. Cross-reactions of these antisera with other antigens, sharing similar amino acid sequences, cannot be completely excluded but is highly unlikely.
NADPH diaphorase activity. The tissue sections were incubated with 1 mM ß-NADPH and 0.5 mM nitro blue tetrazolium, and dissolved in 50 mM tris(hydroxymethyl) aminomethane (Tris)- HCl buffer (pH 8.0) containing 0.2% Triton X-100, for 60 min at 37°C [13]. The tissue sections were then rinsed in PBS and mounted in Kaiser´s glycerol gelatin.
Routine histology. For examination of tissue morphology tissue sections were stained with hematoxylin and eosin.
All sections were examined at the light microscopic level in an Olympus OMX60 system fluorescence microscope equipped with epi-illumination and filter settings for FITC-immunofluorescence. Images of the sections were processed digitally using Adobe Photoshop software. Only contrast and brightness of the images were adjusted.

Chemicals
ß-NADPH, nitro blue tetrazolium, Tris were bought from Sigma Chemical Company (St Louis, MO, USA) and Kaiser´s glycerol gelatin, p-phenylenediamine, and Triton X-100 were aquired from Merck (Darmstadt, Germany).

Calculations
Data are presented as mean, median and range when applicable. The standard deviation of the measurement error/mean (coefficient of variation) was calculated for NO-concentration data from the nasal cavity.

RESULTS

Sinus and Nasal NO concentrations

All but one patient (patient no 2) had the highest individual concentration of NO in their maxillary sinus. The lowest concentration was found in the nasal air in four patients, and in the sphenoidal sinus in the remaining six patients, Table 2. There was a wide range in NO concentration between the patients. We therefore chose to compare the individual NO sinus concentrations found in the nose, and in the sphenoidal sinus to the concentration found in the maxillary sinus (% NO of maxillary sinus NO). The nasal cavity and the sphenoidal sinus had a NO concentration which was less than half of the maxillary sinus in the majority of patients, Table 3. The standard deviation of the measurement error/mean (coefficient of variation) was 12% for NO in nasal cavity.

Localization of different isoforms of NOS

NADPH-diaphorase activity

We found an intense NADPH diaphorase activity in the epithelium in all biopsies from all patients. A similar staining pattern was seen in the mucosa of the sphenoidal and maxillary sinuses and the ethmoidal cells. The apical part of the epithelium had a stronger activity than the basal part. The ciliated cells showed a more intense staining than the goblet cells and basal cells, Figure 1D-F.

NOS1-immunoreactivity

In the sphenoidal sinus, NOS1-immunoreactivity was found in the subapical part of the ciliated cells. The same distribution pattern for NOS1-immunoreactivity was found in the mucosa of the maxillary sinus and the ethmoidal cells, Figure 1G.

NOS2-immunoreactivity

Immunoreactivity against inducible NOS2 was similar to that seen with NADPH diaphorase activity. However, NOS2-immunoreactivity was not demonstrated in that large extent of cells as NADPH diaphorase. NOS2-immunoreactivity could be demonstrated in the sphenoidal and maxillary sinuses and ethmoidal cells throughout the mucosa in mainly the apical but often also the basal part of the ciliated cells, Figure 1H.

NOS3-immunoreactivity

NOS3-immunoreactivity was demonstrated exclusively in the most apical part of the epithelium. No overt difference between different sinus locations was seen, Figure 1I.

DISCUSSION

NO and NOS have previously been shown to exist in the paranasal sinus [4, 10]. However, this is the first report demonstrating the expression of NOS and the production of NO in the sphenoidal sinus. We found the presence of immunoreactivity against all the three major subtypes of NOS, neuronal, inducible and endothelial NOS in the sphenoidal mucosa. This staining pattern was very similar to that found in the ethmoidal cells and the maxillary sinus. Moreover, this study reveals a difference in NO production between the sphenoidal and the maxillary sinus. Possibly this difference reflects a more profound role for NO in the maxillary sinus.

Table 2. NO in the paranasal sinus.
Most samples were taken only once, but when repeated samples were taken, we present these as mean (both values).

Pat id	sex	age	NO in maxillary sinus (ppb)	NO in sphenoidal sinus (ppb)	% NO of maxillary NO	NO in nasal cavity (ppb)	% NO of maxillary NO
1	F	57	7300	1600	22%	2250 (2200, 2300)	31%
2	F	51	160	283	177%	32 (20, 44)	20%
3	F	82	6098	1060	17%	2777 (2673, 2882)	46%
4	M	52	2140	1048	49%	625 (514, 790)	30%
5	F	57	11900	5501	46%	8675 (8650, 8700)	73%
6	M	62	4487	274	6%	1052 (980, 1124)	23%
7	F	15	4167	1643	39%	1374 (1218, 1530)	33%
8	F	48	5401	1555	29%	2883 (2810, 2956)	53%
9	M	58	21875	12035	55%	4043 (3441, 4645)	18%
10	F	67	4392	750	17%	2352 (2120, 2583)	54%

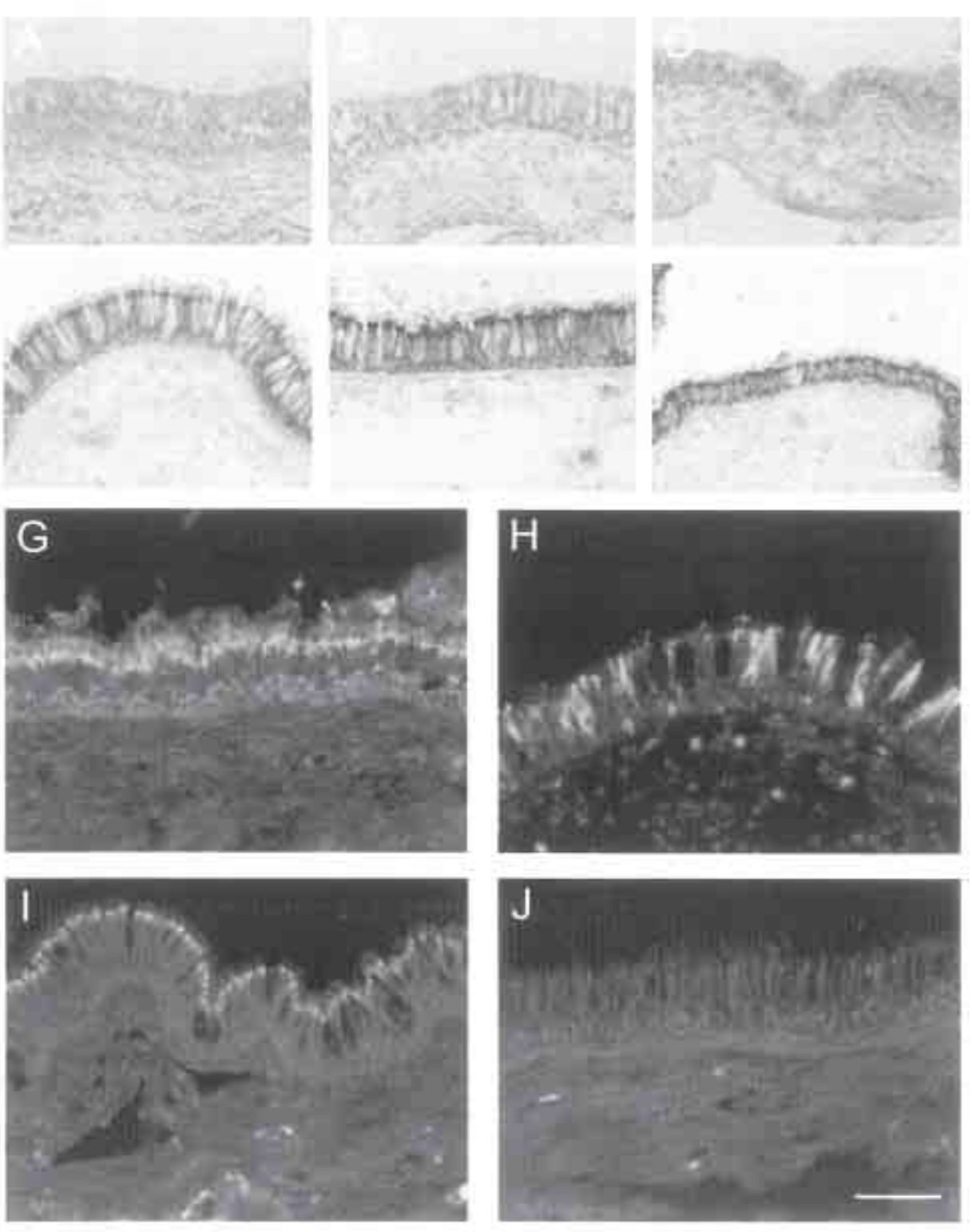

Figure 1. The mucosa of the maxillary sinus, the ethmoidal cells and the sphenoidal sinus as demonstrated by light microscopy (A-F) and immunohistochemistry (FITC immunofluorescence; G-J). (A-C) Hematoxylin staining of (A) the maxillary sinus, (B) the ethmoidal cells and (C) the sphenoidal sinus. (D-F) NADPH diaphorase staining of (D) the maxillary sinus, (E) the ethmoidal cells and (F) the sphenoidal sinus. (G) represents NOS1-immunoreactivity in the sphenoidal sinus, (H) NOS2-immunoreactivity in the sphenoidal sinus and (I) NOS3-immunoreactivity in the ethmoidal cells. (J) represents control, a section from the sphenoidal sinus only incubated with secondary antibodies. The bars represent 100 μm, the bar in F is valid for (A-F) and the bar in J is valid for (G-J).

Table 3. Mean and median values of NO concentration (ppb) in the nasal cavity and the paranasal sinus.

	Nasal cavity	Maxillary sinus	Sphenoidal sinus
Mean	2744	6792	2575
median	2301	4994	1308
(range)	(32 - 8675)	(160 - 21875)	(274 – 12035)

NO is a gas that is produced in many parts of the body, including the paranasal sinus, and exerts many different biological effects. It is well-accepted that NO has toxic effects on microorganisms, such as bacteria, viruses and fungi, and NO has been demonstrated in a higher concentration in the maxillary sinus than in the nasal cavity [10]. The high levels of NO in the maxillary sinus are considered the main reason why these sinuses normally are sterile and that the NO diffuses into the nasal cavity, preventing local infections [10]. Infections do occur in the paranasal sinus, most commonly in the maxillary sinus. Sphenoidal sinusitis, however, only occurs in 16% of all acute sinusitis cases [11]. Our hypothesis was that this might be explained by a higher concentration of NO in the sphenoidal sinus. However, this was not the case, as the NO concentration was about half of that in the maxillary sinuses.

We report a mean maxillary sinus NO concentration value of 6792 ppb. Two of the patients had values over 10 000 ppb. These findings correspond well with the findings reported by Andersson and co-workers [14]. We also found a large difference in NO concentration between patients. The variations in NO-concentration between the different sinuses within the same patient were usually similar, i.e. the nasal and sphenoidal cavity had a NO concentration that was less than half of the maxillary sinus in most patients. In our study, patients were masked during induction of anesthesia and then intubated for at least 30 minutes before NO-measurement started, providing conditions where nasal ventilation has minimal influence on NO levels.

In all patients nasal air samples were taken twice, with a one-minute interval. The variation in NO concentration between the two samples was usually very discrete, which could indicate a quick production of NO from the mucosa. These findings are in accordance with those of Lundberg and co-workers who found a continuous NO production in maxillary sinus [4].

The pattern for immunostaining of NOS was similar in the sphenoidal sinus compared with the maxillary sinus and the ethmoidal cells. As would be expected we found strong staining for NADPH diaphorase and immunoreactivity against the inducible isoform of NOS, NOS2, in the sphenoidal and maxillary sinus, and the ethmoidal cells. NADPH diaphorase is used to demonstrate possible sites of NO expression not discriminating between different isoforms of NOS. Thus, both NADPH diaphorase and NOS2 could be used as markers of the existence of inducible NOS in tissue sample [10]. NOS2 increases when there is an increase of bacteria/ cytokines in the tissue [10]. However, none of our patients had any clinical symptoms of infection or allergic reactions. In the examined mucosal biopsies also NOS1- and NOS3-immunoreactivities could be observed. The function of these NOS isozymes in the paranasal sinus is at present not clear.

The NO air samples were collected from a rather heterogeneous group of patients. Many external factors are known to affect NO production [15]. Smoking is considered to increase NO in some patients, but in other patients it is considered to have no effect on NO concentrations at all [16, 17]. We did not find any difference in nasal NO levels when comparing smokers and non-smokers, however, only one patient admitted smoking. Moreover, the use of highdose preoperative glucocorticoids did not markedly inhibit nasal NO-production, which is in agreement with a previous investigation [18]. Interestingly, we found that there was a large inter-individual difference in NO concentration in the sphenoidal sinus. Reasons for this difference is so far unclear, however none of the patients had any clinical signs of ongoing common cold or recurrent sinusitis.

The surgical approach used in this study, results in that the sphenoidal sinus is ‘dewalled’ and turned into a shallow cave in the nasopharynx. In an ongoing study we try to evaluate if this change in anatomy alters the NO-production in the sphenoidal mucosa.

In conclusion, NO production is present in the sphenoidal sinus. We found NO concentrations in the sphenoidal sinus to be less than half of that in the maxillary sinus. Despite lower concentrations of NO, the sphenoidal sinus is rarely affected by infection. All three NOS were constitutively expressed in the spenoidal sinus mucosa. The reason for this is not clear, but could possibly reflect different functions for NO in this tissue.

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