

Increased nitric oxide production in nasal epithelial cells from allergic patients – RT-PCR analysis and direct imaging by a fluorescence indicator: DAF-2 DA*

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Summary

Background Nitric oxide (NO) is believed to participate in the regulation of airway clearance and non-specific cellular immunity. Recent studies have suggested that airway epithelial cells of allergic and non-allergic individuals may differ in their ability to produce this molecule.

Objective The aim of this study was to detect the difference in NO production in human nasal epithelial cells between normal subjects and patients with perennial allergic rhinitis (AR), and to assess the relationship between the expression of nitric oxide synthase (NOS) isoforms and the severity of the disease.

Methods Nasal epithelial cells were obtained from the inferior turbinate. The expression of mRNAs encoding constitutive endothelial NOS (eNOS) and inducible NOS (iNOS) was studied by reverse transcription-polymerase chain reaction (RT-PCR). Direct NO production in living cells was visualized and quantified by a fluorescent indicator, DAF-2 DA.

Results RT-PCR analysis demonstrated that AR patients with a RAST score of 5 or 6 showed significant increases in the levels of iNOS mRNA and slight reductions in those of eNOS mRNA. Patients with a RAST score of 2–4 also revealed the same tendency however, the difference was not significant. DAF-2 DA imaging demonstrated that epithelial cells, especially the ciliated cells, produced a larger amount of NO than non-epithelial inflammatory cells. Preincubation with L-NAME resulted in an approximate 40% decrease in both groups.

Conclusion These results directly indicate that nasal epithelial cells of AR patients overall produce higher levels of NO through the concomitant expression of different NOS isoforms. Continuous NO production by the epithelial cells in normal subjects further support the hypothesis that NO derived from epithelium may play dual roles in the regulation of nasal airway clearance and in the host defense. In addition, the use of DAF-2 DA provides a reliable method to visualize and quantify the direct NO production of living cells.

Keywords: Nitric oxide synthase, allergic rhinitis, nasal epithelial cells, inferior turbinates, DAF-2 DA, laser scanning confocal microscopy

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Introduction

A significant role for nitric oxide (NO) has been demonstrated in the regulation of mammalian airway function [1–3]. The well-known effects of NO on smooth muscle, neutrophils, lymphocytes, and endothelial and

epithelial cells indicate that NO might be a mediator of major importance in the human respiratory system, particularly in association with inflammatory airway diseases such as asthma [4–8]. In this sense, the respiratory epithelium has been considered to play a pivotal role in NO-mediated inflammatory reactions as a dominant source of NO production. Strong nitric oxide synthase (NOS) activity has been reported in lung tissue preparations as well as in human bronchial epithelial cells [9,10]. Nitric oxide could have beneficial effects on the airway by relaxing airway smooth muscle. Nijkamp *et al.* [5] reported that the inhibition of histamine-induced bronchoreactivity was epithelium-dependent and appeared to be mediated by NO. On the other hand, NO has been shown to play a decisive role in inflammatory tissue injury because of its cytotoxic and immunoregulatory properties when synthesized at high concentrations [4,11].

The cellular origin of NO in the nasal airway is still controversial, and the importance of NO as a potential contributory factor in allergic rhinitis (AR) has yet to be established. Recent studies have shown that a large amount of NO is continuously produced in the nasal airway, and surface epithelial cells of human paranasal sinuses seem to be the dominant source of local NO production [1,12,13]. The primary function of a well-differentiated ciliated epithelium in the nose is to eliminate mucus-trapped micro-organisms, debris, and foreign particles through the beating action of the cilia. The factors regulating ciliary activity are complex, and whether the involvement of NO occurs in this mechanism is not well understood.

We have previously reported differences in NOS isoform levels constitutively expressed in nasal epithelial cells (NECs) from allergic and non-allergic subjects by immunocytochemistry [14]. In the present study, we have extended the previous knowledge and tried to find out whether the severity of the disease may influence the proportion and levels of different NOS isoform expression. For this purpose, reverse transcription-polymerase chain reaction (RT-PCR) was employed to detect a panel of different mRNAs in freshly isolated human nasal brushing cells. In addition, we applied a new fluorescent membrane-permeable indicator, 4,5-diaminofluorescein diacetate (DAF-2 DA), to visualize and quantify the direct NO production of the sampled cells [15,16]. We performed visualization of the NO production with this new fluorescent indicator in combination with laser scanning confocal microscopy (LSCM), and analysed the cellular functions of NO directly on the basis of digital fluorescent images.

Materials and methods

Preparation of nasal epithelial cells

The study was carried out on a group of 40 patients with

symptoms of perennial allergic rhinitis (24 men and 16 women, mean age 26 years) and 13 healthy normal controls (10 men and 3 women, mean age 36 years). The patients with AR had experienced their symptoms for at least 1 years. The diagnosis of AR was defined as the presence of a positive skin test response as well as a positive nasal provocation test to house dust (Torii & Co. Ltd, Tokyo, Japan). All AR patients had a score of 2 or more on a radioallergosorbent test (RAST) to *Dermatophagoides farinae* as measured by the Pharmacia CAP system. For RT-PCR analysis, the AR patients were subdivided into two groups based on the results of RAST score; a group of 17 patients with a RAST score of 2–4 (RAST 2–4), and a group of 23 patients with a RAST score of 5 or 6 (RAST 5–6). Neither patients nor controls had used any topical steroid or other medication for at least one month before the study. The control individuals had no history of chronic nasal symptoms and had normal rhinoscopic appearance. The study was carried out in compliance with the principles of the local ethics committee at the University Hospital, Hiroshima. All subjects provided written informed consent to participate in this study.

To prepare for cell collection, a topical anaesthetic and decongestant were applied to the nose, and secretions or crusts were carefully removed. Cells were then obtained by vigorous scraping of the medial wall of the inferior turbinate under direct vision using a Cytobrush® (Meds-cand Medical, Malmö, Sweden). The cells were placed immediately in chilled Dulbecco's modified Eagle medium (D-MEM; Gibco BRL, Grand Island, NY, USA) containing antibiotics. Part of the samples was prepared for cytocentrifuging to determine the cell proportion by haematoxylin eosin (HE) staining.

Semi-quantification of PCR products and analysis of mRNA expression

Nasal brushing samples were washed in ice-cold Hanks balanced salt solution (HBSS) and flash frozen in liquid nitrogen for RT-PCR analysis. Total cellular RNA was extracted by TRIZOL® reagent (Gibco BRL) according to the methods supplied by the manufacturer. The resulting RNA was quantified using a spectrophotometer. Five micrograms of the cellular RNA was then reverse-transcribed to cDNA in the presence of Moloney murine leukaemia virus reverse transcriptase (2 U/μL, Toyobo, Tokyo, Japan), 1 mM of dNTPs, RNase inhibitor (0.5 U/μL), 50 pmols of random primers, and RT buffer to a total volume of 40 μL. The RT mixture was incubated for 10 min at 30 °C for initial annealing, followed by 20 min at 42 °C for RT and 5 min at 99 °C for inactivation.

An aliquot of the RT product was subjected to PCR amplification in the presence of a mixture containing PCR

buffer, 1.5 mM of MgCl₂, 0.2 mM of dNTPs, 1.25 U of rTaq polymerase (Gibco BRL), and paired primers for each NOS isoform (10 pmoles of each primer) to a total volume of 50 µL. The oligonucleotide primers for human hepatocyte inducible NOS (iNOS): sense primer, 5'-ATGCCA GATGGCAGCATCAGA-3'; anti-sense primer, 5'-TTT CCAGGCCCATTCCTCCTGC-3' (expected product size (S) = 394 base pairs (bp)), and human endothelial NOS (eNOS): sense primer, 5'-CCAGCTAGCCAAAGTCAC CAT-3'; anti-sense primer, 5'-GTCTCGGAGCCATA CAGGATT-3' (S = 672 bp), were customarily prepared from regions where the nucleotide sequences were not conserved among the different isoforms based on the published DNA sequences [17–19]. To check the efficacy of the amplification, primers specific to β-actin: sense primer, 5'-AAGAGAGGCATCCTCACCT-3'; and anti-sense primer, 5'-TACATGGCTGGGGTGTGAA-3' (S = 218 bp), were also prepared [20]. The PCR conditions were as follows: denaturation for 45 s at 94 °C, annealing for 45 s at 55 °C for eNOS and 60 °C for iNOS and β-actin, and extension for 60 s at 72 °C, followed by 20 min at 72 °C for final extension. In order to find the optimal number of PCR cycles for semiquantitative analysis, PCR amplification was carried out in different cycles for each pair of primers. The optimal number of cycles for β-actin, iNOS, and eNOS were determined to be 30, 45, and 45, respectively, using an MJ Research PTC-100 thermal cycler (Watertown, MA, USA). PCR products (10 µL) were analysed in 1.5% agarose gels containing 1 µg/mL ethidium bromide in Tris acetate EDTA (TAE) buffer. After brief washing in TAE buffer, the intensity of the ethidium bromide luminescence was measured with a CCD image analyser (AE-6915, Atto, Tokyo, Japan). The fluorescence intensity of NOS isoforms in each sample was expressed as the ratio relative to the intensity of β-actin in the same sample [21].

Biological NO imaging by DAF-2 DA

In this experiment, cell samples were collected by nasal brushing from 7 normal subjects and 7 AR patients. The AR patients had a RAST score of at least 3. The cells were disaggregated for 60 min at 37 °C in D-MEM containing 0.1% collagenase (Boehringer Mannheim, Mannheim, Germany) and 0.1 µg/mL deoxyribonuclease (Sigma, St Louis, MO, USA) and then washed once in HBSS. Cell viability exceeded 95% as determined by trypan blue dye exclusion. The ciliated cell-rich aggregates were resuspended in HBSS at a final density of approximately 1×10^5 cells/mL and were seeded in poly-L-lysine-coated 24-well culture plates (Sumiron, Tokyo, Japan). Cell labelling was achieved by incubating the cells with 10 µM of DAF-2 DA (Daiichi Chemicals Co., Tokyo,

Japan) in HBSS for 20 min at 20 °C in darkness. The incubation time and the concentration of DAF-2 DA were based on preliminary measurements and on published data [15,16]. To activate NO production, L-arginine was added to the solution at a final concentration of 0.5 mM. In some experiments, a NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME, 1 mM), was administered at the time of dye loading.

Direct visualization of the NO production with this fluorescent indicator was performed in combination with a laser scanning confocal microscope (LSCM, Leica TCS-NT, Heidelberg, Germany) equipped with an argon-krypton laser at an excitation wavelength of 488 nm and a band-pass filter for 500–550 nm. Simultaneous visualization of cell morphology by differential interference contrast (DIC) microscopy allowed us to select epithelial-rich areas in the cell samples. The fluorescence image was obtained as a 1024 × 1024 pixel frame. All other settings, including scanning speed, pinhole diameter, and voltage gain, remained the same for all experiments. The images were stored on the magneto-optical storage devices. Quantitative analysis of nasal brushing cells was based on the fluorescence data that lay in the linear portion of the time course curve. Fifty epithelial cells were randomly selected 10 min after L-arginine administration in each time course experiment and the relative fluorescence intensity was calculated with the 'TCS-NT' software package for Windows NT as previously described [14].

In order to confirm epithelial NO production, we employed double-labelling immunohistochemistry with cytokeratin in some specimens which had been loaded with DAF-2 DA. Loaded specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min at 20 °C. Staining with anticytokeratin antibodies (C-11, Sigma) was performed with tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (R270, DAKO, Glostrup, Denmark) to identify epithelial cells.

Statistical analysis

The comparison of mRNA expression levels for each NOS isoform between the groups was performed with the Mann–Whitney *U*-test because they distributed non-normally. For analysis of DAF-2 DA intensity levels, the Student's *t*-test was used for non-paired comparison of normally distributed data.

Results

RT-PCR analysis of NOS isoform mRNA transcripts

In order to examine the degrees of NOS isoform expression

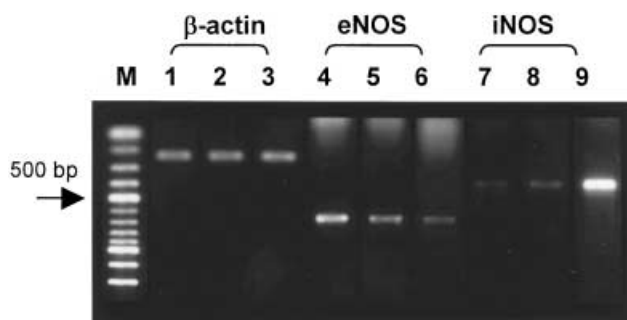


Fig. 1 RT-PCR analysis of β -actin (218 bp), eNOS (672 bp), and iNOS (394 bp) transcripts in the nasal brushing cells. Lanes 1, 4 and 7, cells collected from a normal control; lanes 2, 5 and 8, cells from a patient in the RAST 2–4 group, lanes 3, 6 and 9, cells from a patient in the RAST 5–6 group. M, 100 bp DNA marker.

in nasal brushing samples, we employed semiquantitative RT-PCR analysis in the cells obtained by nasal brushing. Figure 1 shows a representative profile of eNOS and iNOS transcripts for a control subject and for AR patients. Expression levels of β -actin mRNA from each sample were found to be about the same from sample to sample, according to ethidium bromide staining. Primers specific for each NOS isoform yielded the expected products. Figure 2 summarizes the degree of calculated relative ratios of the NOS isoforms in the three groups. Endothelial NOS mRNA transcripts were demonstrated in all samples, with

amplification products seen at the predicted size. AR patients in the RAST 5–6 group showed reductions in eNOS mRNA expression compared with the control group ($P = 0.039$), but there was no significant difference between the RAST 2–4 group and the control group. On the other hand, AR patients generally showed relatively high mRNA levels for iNOS from untreated lesional inferior turbinates, whereas those in control subjects were weakly detectable. There was significant difference in iNOS ratios between the control subjects and the AR patients in the RAST 5–6 group ($P = 0.0021$).

Direct imaging of NO production by DAF-2 DA

In order to determine cell types as the major source for NOS expression in nasal brushing preparations, as well as to examine contamination of the epithelial preparation by RNA from other minor cell types, we applied DAF-2 DA for direct bioimaging of NO production. Brushing cells sampled from AR patients tended to reveal an increase in the proportion of inflammatory cells, e.g. lymphocytes, eosinophils, and neutrophils. The percentage of eosinophils, as assessed by HE staining, was higher in the RAST 5–6 group ($9.56 \pm 11.16\%$) compared to that in the control group ($0.36 \pm 0.51\%$) or in the RAST 2–4 group ($1.79 \pm 1.54\%$).

Figure 3 shows representative LSCM images triggered

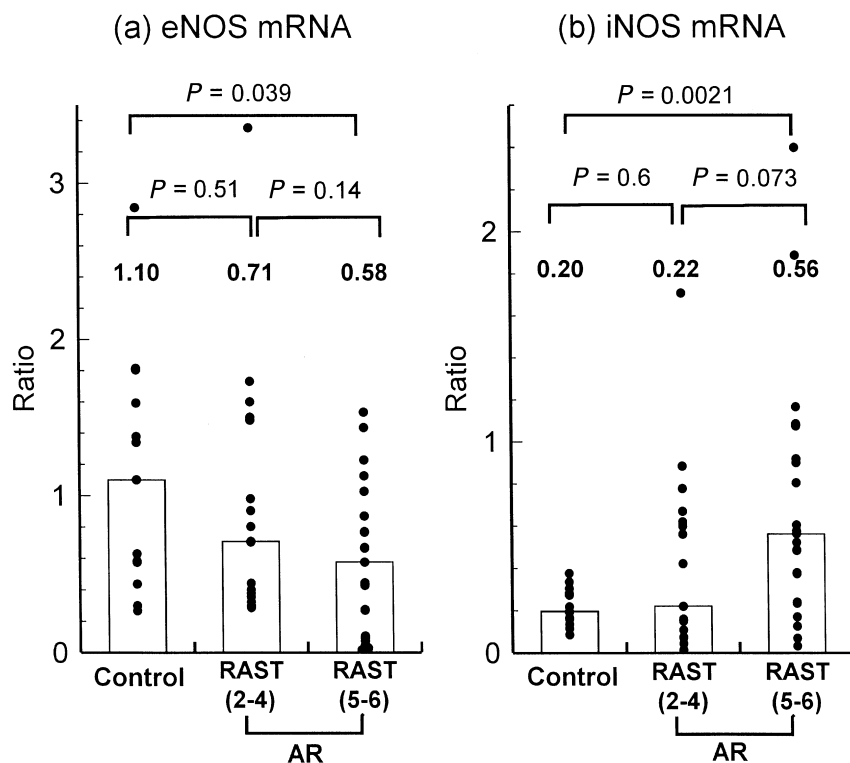
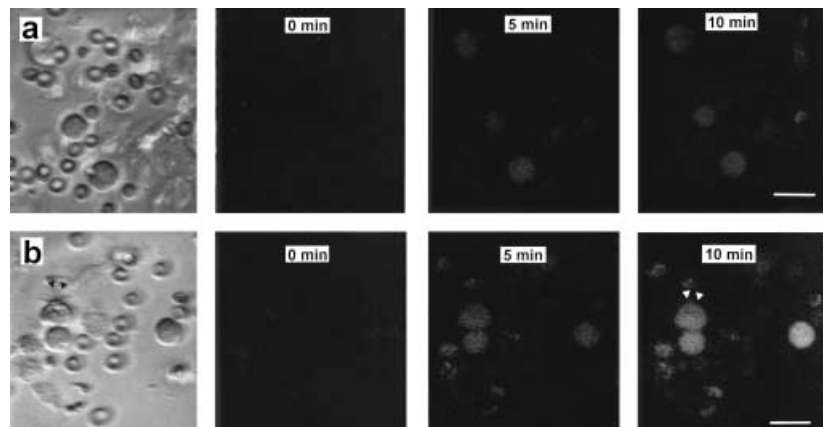


Fig. 2 Relative mRNA expression levels of eNOS (a) and iNOS (b) in nasal brushing cells sampled from normal control subjects ($n = 13$), AR patients in the RAST 2–4 group ($n = 17$), and AR patients in the RAST 5–6 group ($n = 23$). The levels are expressed as ratio relative to those of β -actin using RT-PCR. Columns represent median values.

Fig. 3 Time course and distribution of NO production detected by using DAF-2 DA fluorophore in nasal brushing cells from a normal control subject (a) and an AR patient (b). The left panels correspond to differential interference contrast (DIC) photomicrograph. Sequence of DAF-2 DA fluorescence images was obtained from the identical site at intervals between 0 and 10 min after stimulation with 0.5 mM L-arginine. Distinct DAF-2 DA reactivity is found to be localized in the ciliated epithelial cells (arrowheads). All images were obtained by LSCM under the same hardware and time course settings. Bar = 5 μ m.



by the reaction between DAF-2 DA and generated NO. Morphological evaluation using DIC microscopy clearly reveals epithelial ciliated cells by their relatively large size and characteristic brush borders. At the baseline period, only a weak, diffuse background fluorescence could be observed with no obvious localization of fluorescence within individual cells. After administration of 0.5 mM L-arginine, a gradual increase in intracellular fluorescence was observed, and it became fully apparent within 10 min. The fluorescence intensity of most cells usually reached a plateau at about 20 min after L-arginine administration. Areas of increased fluorescence intensity were most prominent along the brush borders of epithelial ciliated cells. Those cells from the AR patient showed a higher degree of NO production. Some of the small, round-shaped, inflammatory cells also appeared to be fluorescent, though

less so than the epithelial cells. Figure 4 depicts colocalization of DAF-2 DA fluorescence and TRITC-labelled cytokeratin immunoreactivity. We found that the spatial resolution of DAF-2 DA was still maintained after double-labelling immunohistochemistry. Cells that showed distinct green fluorescence for NO production correlated well with the distribution of cytokeratin.

The column-bar graph in Fig. 5 summarizes the degree of DAF-2 DA fluorescence intensity 10 min after L-arginine administration. Epithelial cells prepared from the AR patients showed significantly higher degrees of the fluorescence intensity than did those from the control subjects ($P < 0.01$). Fluorescence intensity augmentation was uniformly suppressed in both groups when the cells were preincubated with 1 mM L-NAME, a non-specific NOS isoform inhibitor, prior to L-arginine administration. The average degrees of suppression were 46% in the controls and 40% in the AR patients, and no significant difference was observed between the groups.

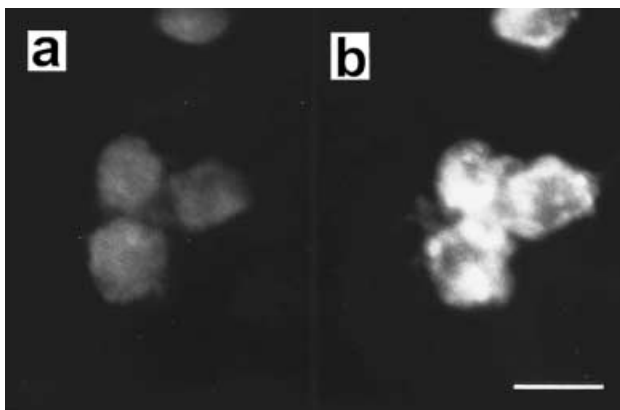


Fig. 4 LSCM photomicrographs showing NO production by nasal epithelial cells. Specimens which had been loaded with DAF-2 DA (a) were fixed and stained with TRITC-labelled anti-cytokeratin antibodies (b). The distribution of DAF-2 DA reactivity is found to be localized in these cytokeratin positive epithelial cells. Bar = 5 μ m.

Discussion

Recently, considerable attention has focused on the ability of NECs to produce NO [1,12]. Nitric oxide derived from the airway epithelium may play dual roles in the regulation of airway clearance and in host defense [2,9]. This raises the possibility that NECs can produce varying amounts of NO throughout the regulation of different NOS isoform expression in allergic conditions.

Using the RT-PCR technique, we analysed the profiles of eNOS and iNOS isoform gene expressions in 13 normal subjects and 40 AR patients with apparent clinical symptoms. The AR patients were further subdivided into two groups based on the results of RAST score. In this study, we chose RAST scores as an objective parameter instead of daily nasal symptoms. It is possible to consider

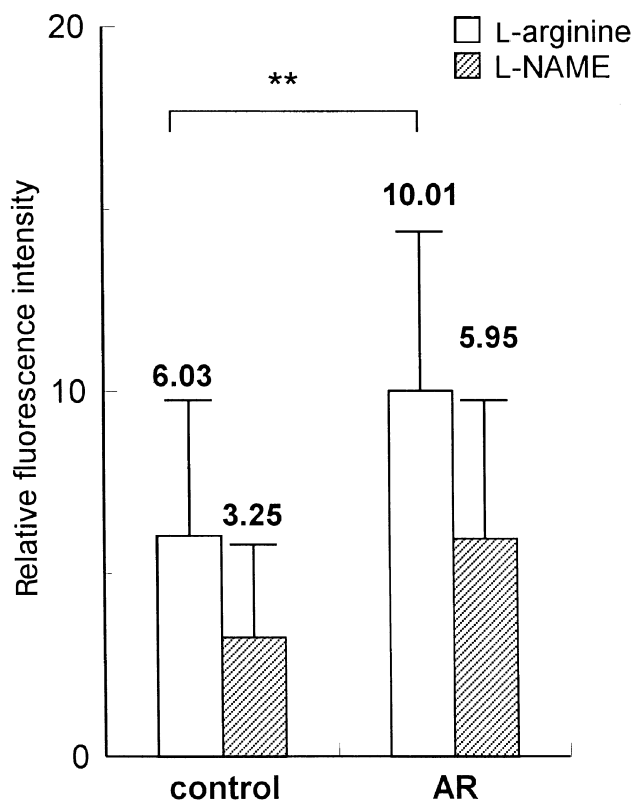


Fig. 5 DAF-2 DA fluorescence intensity of NECs measured by LSCM. Fifty epithelial cells were randomly selected in each subject on the basis of morphology. The cells were stimulated for 10 min either with 0.5 mM L-arginine alone or with a combination of 0.5 mM L-arginine and 1 mM L-NAME. Each column represents the mean $\pm$ SEM of 350 epithelial cells. $**P < 0.01$.

that a higher level of RAST score usually indicates evidence of severe allergy, because allergen specific IgE antibodies play a pivotal role in mediating allergic reactions [22]. In our study population, no quantitative difference could be seen in the degree of constitutive eNOS expression between the control subjects and AR patients in the RAST 2–4 group, whereas patients in the RAST 5–6 group have shown relative decrease. Significant up-regulation of iNOS transcripts was also observed in this group.

These results are in accordance with our previous reports on the human nose as well as those about human bronchial and lung specimens [5,10,14,23]. One of the constitutively expressed, Ca^{2+} -dependent NOS isoforms first identified in endothelial cells is now known to be more widely distributed in various cells, including respiratory epithelial cells [9,24,25]. Shaul *et al.* [24] investigated the *in vitro* expression of eNOS in NCI-H441 human bronchiolar epithelial cells. Positive immunoreactivity was observed in the cell periphery, consistent with the association of this

isoform with the cellular membrane. Furukawa *et al.* [26] found that distinct immunoreactivity for eNOS was commonly localized to endothelial cells, submucosal glands, and epithelial cells in human nasal inferior turbinate. They reported that immunoreactivity for eNOS in the epithelium had no correlation with the degree of local inflammation. In this study, we also observed eNOS mRNA transcripts in all samples. However, part of the samples in the severe AR group revealed weakly detectable eNOS mRNA expression. This is in contrast with our previous immunohistochemical findings that showed no significant difference in eNOS protein expression between control subjects and AR patients [14]. We examined cytocentrifuge preparations in specimens and excluded the possibility that this isoform was derived from endothelial contaminants. We assume that the reduction in eNOS mRNA observed in this study may be due to a relative decrease in the proportion of epithelial cells obtained by nasal brushing. Another interpretation is possible that functional damage to the surface ciliated epithelium is caused by chronic allergic inflammation in the RAST 5–6 group.

Hepatocyte NOS was the first inducible member of the NOS family cloned from humans [17]. The high-level tonic expression of iNOS and NO production by respiratory epithelial cells has been commonly reported in asthmatic patients [4,7,8,11]. Ramis *et al.* [27] reported that nasal polyps in the human nose contained higher levels of total NOS activity than did nasal mucosal tissues. In addition, nasal polyps mainly contained iNOS activity, whereas all NOS activity detected in the nasal mucosa was in constitutive form. In both cases, NOS activity was mainly localized in the epithelial layer. The present findings also demonstrated that high levels of iNOS mRNA expression were generally observed in AR patients, being more augmented in the severely diseased population. In this sense, differential activity of NOS in human nasal mucosa should be considered.

In allergic rhinitis, there is an influx of inflammatory cells into the mucosa layer. These cells produce various cytokines, such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and $\text{IFN-}\gamma$, all well-established stimulators of iNOS gene expression [28,29]. Thus, the increases in iNOS mRNA seen in this study may result from direct induction by these inflammatory cytokines in an autocrine and/or paracrine fashion. In addition, the coexistence of constitutive and inducible NOS in human NECs may reflect a complex mechanism promoted by surface epithelial cells to protect the host from noxious assault as well as to prevent the induction of airway hyper-reactivity.

Although RT-PCR is a sensitive assay, it is difficult to determine whether the assay is measuring mRNA for each NOS isoform in epithelial cells or other non-epithelial origin cells. In order to answer this question, we applied an

indicator, DAF-2 DA, to the sampled cells for bioimaging of NO. This fluorescent indicator provided direct evidence of NO production by nasal epithelial cells, especially by differentiated ciliated cells. DAF-2 DA can be loaded into cells for temporal and spatial analysis of NO production, like Ca^{2+} indicators. In the presence of dioxide under neutral pH, the relatively non-fluorescent DAF-2 DA reacts rapidly with NO irreversibly inside the cell to yield its highly fluorescent product triazolo fluorescein (DAF-2 T) [15,16]. Other stable oxidized forms of NO, such as NO_2^- and NO_3^- , and other reactive oxygen species, such as O_2^- , H_2O_2 , and ONOO^- , do not react with DAF-2 DA to yield any fluorescent product. Time dependent changes of DAF-2 intensity seem to rely on the cell types and equipments used for image analysis [30,31]. In this study, we found that a 10 min time point was suitable to obtain the most informative data. Employment of the LSCM also made it possible to obtain thin optical sections and enabled us to measure DAF-2 DA fluorescence intensity without interference by out-of-focus noise derived from the media.

We found that NECs in AR patients generated a large amount of NO as clearly indicated by increased levels of DAF-2 DA fluorescence. Thus, while a fraction of increased iNOS gene expression in AR patients might have reflected infiltrating macrophages and other inflammatory cells mRNA, the majority must have primarily reflected augmented gene expression for iNOS in the epithelial cells. In addition, even in normal subjects, most epithelial ciliated cells revealed distinct intracellular fluorescence. It is of most interest that most subjects in the control group demonstrated low iNOS mRNA expression in contrast with distinct eNOS messages. Together with the fact that eNOS activity is mainly regulated by calcium influx rather than at the level of transcription, we speculate that the nasal ciliated cells can produce a relatively high amount of NO only by single expression of a constitutive type of NOS. Previous reports concerning an NO-dependent control mechanism for regulating the ciliary beating activity also support this idea. The ciliary activity is physiologically regulated by rapid fluctuations of intracellular $[\text{Ca}^{2+}]$, and continuous high-level NO production inside the ciliated cells is tightly associated with this mechanism [32,33]. As a consequence of the effects on ciliary activities, NO modulates mucociliary clearance, an important physical airway defense.

Another noticeable finding is that the failure of total blockade in DAF-2 DA fluorescence following treatment with 1 mM L-NAME. This is in contrast with previous studies which have demonstrated almost total suppression of fluorescence in the mouse macrophage cell line [30] and in slices of rat brain [31]. The exact reason for the difference is unclear. However, we speculate that continuous NO formation coupled with CBF regulation in the

epithelial ciliated cells might be relatively independent of extracellular L-arginine concentration. If the endogenous level of L-arginine is sufficient to support NOS activity, this may account for the fact that NOS inhibitors do not cause complete inhibition of NO formation.

The nasal brushing method yielded a cell population containing a majority of epithelial cells. In this study, we demonstrated that NECs of both normal subjects and AR patients constitutively expressed eNOS mRNA. In addition, increased expression of iNOS mRNA as well as concomitant high NO production in AR patients was observed. The predominant site of production was identified in the epithelial ciliated cells by a new NO indicator, DAF-2 DA. This method in combination with LSCM provides a reliable tool to visualize and quantify the direct NO production of living cells.

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