

ORIGINAL PAPER

The effect of endogenous nitric oxide on mechanical ciliostimulation of human nasal mucosa

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Clinical and Experimental Allergy

Summary

Background Endogenous nitric oxide (NO) production by the inducible NO-synthase is enhanced in the nasal respiratory epithelium of patients with allergic rhinitis. Recent experimental data suggest endogenous NO to be strongly involved in the regulation of ciliary activity, the driving force of the mucociliary transport system.

Objective In this study, we investigated the effect of endogenous NO on mechanical stimulation of ciliary activity in a nasal mucosa explant model.

Methods Cultures of nasal mucosa explants were incubated with TNF- α and bacterial lipopolysaccharides (LPS) to enhance endogenous NO production. Direct *in vitro* NO imaging was performed by the fluorescent NO-indicator DAF-2 DA and laser scanning confocal microscopy. Ciliary beat frequency (CBF) was determined using a photoelectric technique. Mechanical stimulation was performed by two consecutive flow increments in a closed perfusion chamber. Endogenous NO-synthesis was blocked by L-NAME before the second flow stimulation.

Results Under control conditions the mean rise of CBF relative to baseline was 30.2% during the first flow increment and 30.7% during the second flow increment. Blocking of the endogenous NO synthesis in TNF- α /LPS-stimulated cultures reduced baseline CBF by $10.6 \pm 2.1\%$ ($P < 0.05$) but the effect of mechanical ciliostimulation on CBF remained unchanged (36.0% vs. 38.2%).

Conclusion In conclusion, endogenous NO- and Ca^{2+} -dependent mechanical stimulation of ciliary activity probably use independent intracellular signalling pathways. The combination of both effects on ciliary activity is likely to improve the local defence against inhaled allergens in patients with nasal allergies.

Keywords allergic rhinitis, mechanical ciliostimulation, mucociliary transport, nitric oxide

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Introduction

Mucociliary transport is an important defence mechanism of the respiratory mucosa, clearing its surface from inhaled particles. The driving force of this complex system is the ciliary activity of the respiratory epithelium, commonly quantified by the ciliary beat frequency (CBF) [1].

The baseline ciliary activity is physiologically altered by several local and autonomic mechanisms [2]. Among others, endogenous nitric oxide (NO), principally synthesized by the inducible NO synthase (iNOS) within the respiratory epithelium of the nasal sinuses, has been demonstrated to increase baseline CBF of airway epithelia *in vitro* [3, 4]. This is especially interesting in pathologic conditions like in allergic rhinitis (AR) or other types of nasal inflammation. While iNOS is only weakly expressed

in the nasal mucosa of healthy individuals [5, 6] it was found to be strongly enhanced in the nasal respiratory epithelium of patients with AR [7, 8].

NO-dependent base line ciliary activity of respiratory epithelium can be further modified by additional stimuli like acetylcholine [9]. Another powerful mechanism of ciliostimulation is mechanical alteration of the epithelial surface, physiologically caused by the ventilated air stream or inhaled particles [10–13].

Today it is not clear, whether an enhanced endogenous NO synthesis in AR or other types of nasal inflammation only increases baseline ciliary activity, or whether it may also interfere with other regulators of ciliary activity, especially mechanical ciliostimulation.

The aim of the study presented here was to investigate the effect of endogenous NO on mechanical ciliostimulation in human nasal mucosa explants *in vitro*. The results shall further elucidate the pathophysiology of nasal mucociliary transport in AR.

Materials and methods

Tissue preparation and culture

Ciliary activity was investigated *in vitro* in cultured human mucosa explants originating from inferior nasal turbinates of healthy volunteers who underwent surgery for functional improvement of the nasal airflow (septoplasty or septorhinoplasty) including partial resection of the inferior turbinates (turbinoectomy). Informed consent was obtained from all patients before surgery in accordance with the advice of the local ethics committee. Only mucosa explants from patients with no evidence of concomitant allergic, inflammatory, or neoplastic disease of the nose or the nasal sinuses were used.

Thin mucosal strips were dissected from the specimens immediately after surgery, washed in phosphate-buffered saline to remove all blood and debris, and cut into small pieces (1–2 mm²).

Explants were then cultured in Ham's F12-DME 1 : 1 (Sigma, Munich, Germany D 6421) with L-glutamine, supplemented with a final concentration of 100 IU/mL streptomycin and 100 IU/mL penicillin G (pH 7.4), in a humidified air CO₂ incubator (5% CO₂) at 37 °C for a maximum of 7 days. The medium was changed after 1 day and then every other day.

For up-regulation of iNOS, TNF- α (Cell Concepts GmbH, Umkirch, Germany) at a concentration of 1 ng/mL, and lipopolysaccharide (LPS ; bacterial lipopolysaccharides from *Escherichia coli*, Sigma L 2880) at a concentration of 1 ng/mL were added to the culture medium 24 h before the experiments.

In vitro nitric oxide imaging

Direct *in vitro* NO imaging was performed by cell labelling with the fluorescent NO-indicator DAF-2 DA (Daiichi Pure Chemicals, Tokyo, Japan). For this, nasal epithelial cells were harvested by nasal curettage in three healthy volunteers. The obtained cell samples were mechanically disaggregated by shaking of the probes, washed in culture medium, and cultured according to the protocol for the mucosa explants. For up-regulation of iNOS, TNF- α at a concentration of 1 ng/mL, and LPS at a concentration of 1 ng/mL were added to the culture medium 24 h before NO imaging.

For cell labelling, the cells were incubated with 10 μ M of DAF2-DA in culture medium for 20 min at room temperature in darkness based on own preliminary measurements

and on the literature [14]. In some experiments, NO synthase activity was blocked by administration of L-NAME (10⁻² M; Sigma N 5751) at the time of dye loading. Direct visualization of NO production with the fluorescent NO indicator was performed by using a laser scanning confocal microscope (Leica TCS SP; Leica microsystems, Heidelberg, Germany) with an argon-krypton laser at an excitation wave length of 488 nm and a band pass filter for 500–550 nm. Simultaneous visualization of cell morphology was done by light microscopy. By this, clusters of vital, ciliated respiratory epithelial cells (CRECs) were selected for fluorescence imaging. The fluorescence images were obtained as 512 $\times$ 512 pixel frames. Scanning speed, pinhole diameter, and voltage gain remained the same for all experiments. Images were stored on magneto-optical storage devices.

Measurement of ciliary beat frequency

CBF was determined using computerized microscopic photometry [9]. Briefly, a photometer was mounted on a phase-contrast microscope (Zeiss, Oberkochen, Germany) above a two-way beam splitter for simultaneous visual control of the investigated specimen. A diaphragm with a central aperture measuring 0.16 mm in diameter was mounted in the measuring tube, resulting in a measured spot of 2 μ m on the specimen using a 40 $\times$ objective. The variations in light intensity by the beating cilia were recorded by the photometer, filtered, pre-amplified, displayed on an oscilloscope, and digitized by means of an A/D converter. The digitized signal was then analysed online by means of a personal computer. CBF was determined as the first harmonic of a fast Fourier transformation analysis of the recorded signal over a period of 10 s. Measurements were carried out on 10 different sites within the same high-power field. Only cells within morphologically intact ciliated epithelium were examined. For further statistical analysis the mean CBF of each measurement series was used.

Experimental design

After microscopic screening for vital and confluent ciliated epithelium, cultured tissue samples were placed in a closed perfusion chamber constantly perfused by culture medium at a minimal flow rate of 0.5–1 mL/h to prevent accumulation of cellular mediators and to constantly exchange the medium but to minimize mechanical stimulation of ciliary beating. The chamber was mounted on the heated object table of the phase-contrast microscope, thus maintaining a constant temperature of 37 °C. Tissue samples were allowed to equilibrate for 30 min before the first baseline CBF was determined. Only tissue samples with a mean baseline CBF between 7.0 and 11.0 Hz were used.

In a first set of control experiments, the effect of L-NAME on CBF of cultured mucosa explants with and without up-regulation of iNOS was determined.

In a second set of control experiments, cultured mucosa explants were mechanically stimulated by two repeated flow increments with a flow rate of 50 mL/h. Following determination of the first baseline value (baseline 1), CBF was determined again at minutes 1–5 during the first flow increment (flow 1). Flow rate was subsequently reduced to minimal flow rate, and the culture specimens were allowed to equilibrate for 30 min before determination of the second baseline value (baseline 2). Finally, the stimulation procedure was repeated in order to determine a second flow value (flow 2).

In the main set of experiments, 30 min after the first flow increment a new baseline CBF was determined (recovery). Subsequently, the NO synthase activity was blocked by continuous perfusion with L-NAME (10^{-2} M). In order to determine the effect of L-NAME on CBF, a second baseline CBF (baseline_{L-NAME}) was determined after 30 min, before the second flow increment.

Statistical analysis

Values were expressed as mean $\pm$ standard deviation of CBF changes relative to baseline. For statistical analysis the paired *t*-test was applied. To compare the rise in CBF ($\Delta\text{CBF}_{\text{flow}}$) induced by two repeated flow – increments, $\Delta\text{CBF}_{\text{flow}}$ was defined as a quotient of CBF during flow increment through baseline CBF before flow increment. A probability level of < 0.05 was considered to be statistically significant.

Results

In vitro nitric oxide imaging

In order to prove CRECs as a major source for endogenous NO-production, we performed direct *in vitro* NO imaging by the fluorescent NO-indicator DAF-2 DA. Light microscopy allowed identifying clusters of vital CRECs by their characteristic morphology with vigorously beating cilia. Representative laser scanning confocal microscopy (LSCM) images are shown in Fig. 1. After stimulation by TNF- α and LPS a strong enhancement of NO-fluorescence intensity could be found in CRECs, compared with native, unstimulated CRECs. After blocking of endogenous NO production by L-NAME, NO-staining of CRECs was weak to absent. Occasionally, single, not ciliated cells within the epithelial layers (goblet cells) and small, round-shaped, inflammatory cells in the surrounding culture medium also appeared to be fluorescent.

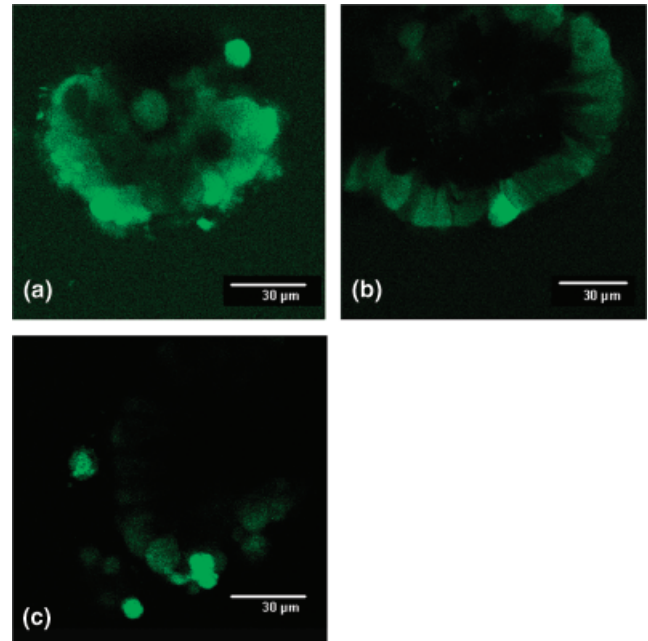


Fig. 1. Representative laser scanning confocal microscopy photomicrographs showing direct *in vitro* nitric oxide (NO) imaging by the fluorescent NO-indicator DAF-2 DA in clusters of ciliated respiratory epithelial cells (CREC; $\times 20$). (a) Strong NO-fluorescence in CRECs after stimulation by TNF- α /LPS. (b) Intermediate NO-fluorescence in native, unstimulated clusters of CRECs. (c) Weak to absent NO-fluorescence in CRECs after blocking of endogenous NO production by L-NAME. Single cells (goblet cells, inflammatory cells) show a persistent NO-enhancement. LPS, lipopolysaccharide.

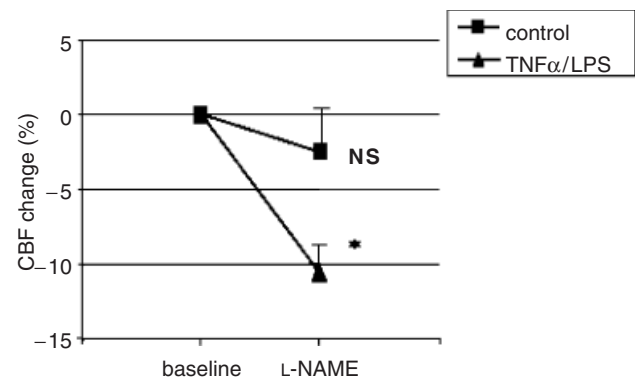


Fig. 2. Effect of endogenous nitric oxide on baseline ciliary beat frequency (CBF) in cultured nasal respiratory epithelium in controls without stimulation ($n = 5$) and after stimulation with TNF- α /LPS ($n = 10$; $P < 0.05$). Mean $\pm$ SD; LPS, lipopolysaccharide.

Effect of endogenous nitric oxide on baseline ciliary beat frequency

The effects of L-NAME on base line CBF are shown by Fig. 2. In controls without TNF- α /LPS stimulation, CBF remained almost constant during perfusion with the specific NOS antagonist L-NAME ($-2.5 \pm 2.9\%$; $n = 5$; NS). In TNF- α /LPS-stimulated culture specimens, CBF

was significantly reduced by $-10.6 \pm 2.1\%$ during perfusion with L-NAME ($n = 10$; $P < 0.05$).

Effect of repeated mechanical stimulation on ciliary beat frequency

The effect of repeated flow increments (50 mL/h) on CBF of cultured respiratory mucosa was investigated in eight control experiments (Fig. 3a). During the first flow increment CBF increased from baseline to $30.2 \pm 6.3\%$ ($P < 0.05$), returned to baseline ($-0.6 \pm 4.2\%$) and increased to $31.3 \pm 9.9\%$ during the second flow increment ($P < 0.05$).

The mean $\Delta\text{CBF}_{\text{flow}}$ was 30.2% during the first flow increment and 30.7% during the second flow increment. There was no statistically significant difference in $\Delta\text{CBF}_{\text{flow}}$ between the first and second flow increments.

Effect of endogenous nitric oxide on mechanical stimulation of ciliary beat frequency

The results of the main experiments ($n = 10$) are shown in Fig. 3b. Baseline CBF (baseline 1) was increased by $36.0 \pm 10.5\%$ during the first flow increment ($P < 0.05$), and recovered to $-0.7 \pm 2.6\%$ within 30 min (recovery). Blocking endogenous NO production by L-NAME significantly reduced CBF to $-11.1 \pm 7.9\%$ as shown above (baseline L-NAME). CBF was again increased to $23.1 \pm 11.4\%$ during the second flow increment ($P < 0.05$).

$\Delta\text{CBF}_{\text{flow}}$ was 36.0% during the first flow stimulation and 38.2% during the second flow stimulation. There were no statistically significant differences in $\Delta\text{CBF}_{\text{flow}}$ before and after blocking of endogenous NO synthesis by L-NAME.

Discussion

We investigated the effect of endogenous nitric NO on mechanical stimulation of human nasal ciliary beat frequency in an *in vitro* model using cultured human nasal mucosa explants.

It is generally accepted that mechanical alteration of ciliated respiratory cells increases CBF. Therefore, in experimental models uncontrolled flow is usually minimized [15]. On the other hand, mechanical stress has been shown to be an important factor for stimulation of ciliary activity *in vitro* [10, 12, 16] and mucociliary function *in vivo* [17].

Nasal respiratory epithelial cells are subjected to cyclic mechanical stress by the nasal air stream and mucociliary transport time was found to have an inverse relationship with the nasal airflow [17]. The main flow stream is passing through the middle meatus next to the ostiomeatal complex [18]. Thus, it seems reasonable that mucociliary transport is physiologically enhanced by mechanical

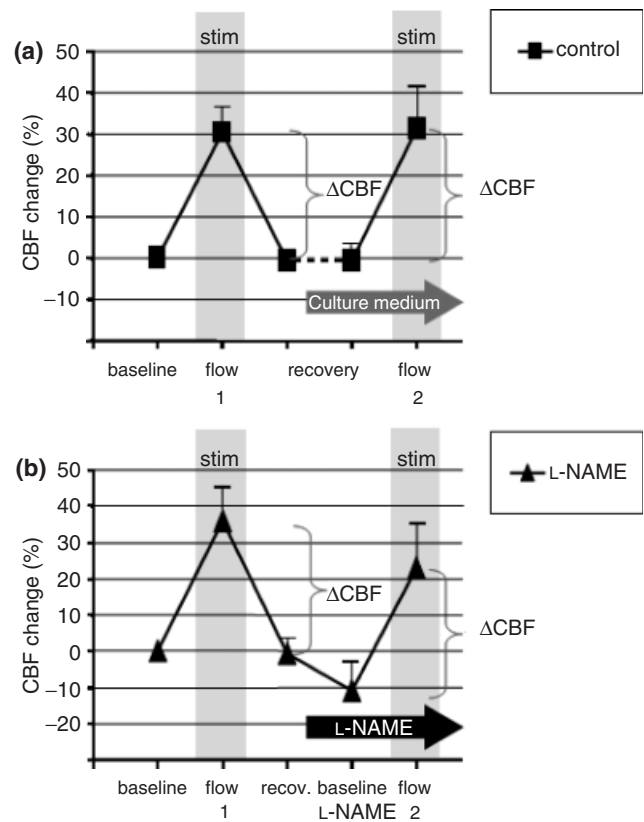


Fig. 3. (a) Control experiments: Effect of two repeated flow increments on ciliary beat frequency (CBF) of cultured nasal mucosa without blocking of endogenous nitric oxide (NO)-production ($n = 8$, mean $\pm$ SD). (b) Main experiments: Effect of two repeated flow increments on CBF before and after blocking of endogenous NO production by L-NAME ($n = 10$, mean $\pm$ SD).

ciliostimulation in this key area of the sinunasal system where the nasal sinuses are draining into the nasal cavity.

Mechanical alteration of single ciliated respiratory cells leads to an increase in cytoplasmic Ca^{2+} due to a combination of extracellular Ca^{2+} influx and an inositol-1,4,5-triphosphate (IP_3) mediated release of Ca^{2+} from intracellular stores both resulting in an acceleration of ciliary beat frequency [19, 20]. In intact respiratory epithelium, the mechanically induced rise of intracellular calcium is propagated to the surrounding adjacent ciliated cells ('calcium signalling') leading to a Ca^{2+} -dependent, coordinated regional stimulation of ciliary activity. Therefore, the use of intact respiratory epithelium is indispensable for the investigation of the complex mechanisms of mechanical ciliostimulation [12].

In the present investigation, we used repeated flow stimuli in a closed perfusion chamber to directly compare the effect of mechanical ciliostimulation within the same specimen before and after experimental intervention. The results of the control experiments confirmed the reproducibility of our model. Furthermore, the use of cultured

human nasal mucosa explants permitted intact respiratory epithelium naturally attached to its basement membrane to be studied close to the *in vivo* situation [9, 21].

In order to create an *in vitro* model of (allergic) rhinitis, endogenous NO production by iNOS was up-regulated by incubation with the proinflammatory substances TNF- α and bacterial LPS [6, 9]. We were able to prove the enhanced NO production within CRECs by direct imaging of NO production by the fluorescent NO-indicator DAF-2 DA. Our findings were well corresponding to the findings of Takeno *et al.* [14] in nasal epithelial cells from allergic patients.

Up-regulation of iNOS expression in cultured respiratory epithelium by TNF- α and/or LPS was demonstrated after 6 h of incubation [6]. Up-regulation of NO-dependent ciliary beat frequency was shown to start after 4 h, remaining on a constantly elevated level for at least 24 h [3]. This makes it possible to investigate TNF- α /LPS-induced ciliary activity within this period [22].

To evaluate NO-depending effects on ciliary activity and its regulatory mechanisms, endogenous NO production was blocked by exposure to the specific NOS-antagonist L-NAME. Our results confirmed that in human nasal mucosa baseline CBF depends on the extent of endogenous NO production and further validated the experimental model.

There are conflicting reports about possible interactions of Ca^{2+} -dependent ciliostimulation and other regulatory mechanisms of ciliary activity, especially the NO-cGMP-PKG pathway [23–25]. In cultured rabbit tracheal epithelium Uzlaner and Priel [24] found a sustained activation of PKG to depend not only on activation of the NO pathway but also on an elevated intracellular Ca^{2+} -concentration. Likewise, Zagoory *et al.* [25] demonstrated intimate interconnections between a rise of intracellular Ca^{2+} , induced by activation of muscarinic receptors in tissue cultures grown from frog oesophagus and palate, and the activation of PKA and PKG with an unimpeded activity of the latter being essential for the CBF enhancement. In contrast, Salathe and coworkers found mechanisms of Ca^{2+} -stimulation of CBF to be independent from activation of PKG and the NO pathway in single ovine airway epithelial cells [26, 27]. More recently, Zhang and Sanderson [28] investigated the role of cGMP, part of the NO pathway and substrate of PKG, in the regulation of rabbit airway CBF. Their results suggest that cGMP regulates rabbit airway CBF in both a Ca^{2+} -dependent manner and a Ca^{2+} -independent manner, and that the NO-cGMP-PKG pathway is not essential for Ca^{2+} -dependent increases in CBF in their model. Unfortunately, this complex and somewhat controversial situation is further complicated by increasing evidence for species differences in the mechanisms of CBF regulation [26].

In our model of cultured human nasal mucosa, we found the extent of mechanical ciliostimulation to be

principally independent from an endogenous NO production. Although we did not specifically address single steps of the pathway, our findings further support the concept, that Ca^{2+} -dependent mechanical ciliostimulation is not closely linked to the NO-cGMP-PKG pathway.

On the other hand, we found the maximum CBF after flow stimulation during unblocked endogenous NO production. This suggests a synergistic effect of Ca^{2+} - and the NO-dependent pathway downstream, possibly on the axonemal level. Ca^{2+} -dependent and cyclic nucleotide-dependent kinases have been demonstrated to phosphorylate different axonemal proteins [28, 29] but further investigations are necessary to confirm this in cilia of human respiratory cells.

In the majority of patients with seasonal or perennial AR, nasal mucociliary clearance is not impaired [30] and the mean velocity of mucociliary transport was demonstrated to be significantly faster than in patients with chronic rhinosinusitis of non-specified aetiology [31]. These findings further support the concept of a NO-dependent enhancement of ciliary activity in inflamed nasal mucosa.

In conclusion, our findings suggest that mucociliary transport may be further enhanced by mechanical ciliostimulation in the presence of increased endogenous NO like in AR. Transferred to the clinical situation, the combination of both effects on the driving force of the mucociliary system is likely to improve the first line local defence against inhaled allergens in patients with nasal allergies.

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