Constitutive nuclear factor-κB activity in human upper airway tissues and nasal epithelial cells


ABSTRACT: Respiratory epithelial cells are actively involved in the host defence and inflammatory reactions of the airways. Nuclear factor-κB (NF-κB) is a transcription factor that plays a pivotal role in many cellular responses to environmental changes. The inducible nitric oxide synthase (iNOS) isofrom has been implicated in airway inflammation as well as in normal airway function. In this study, the hypothesis that NF-κB may be associated with iNOS expression in airway epithelium, not only in inflammatory processes but also under physiological conditions was examined.

NF-κB deoxyribonucleic acid-binding activity was assayed by means of electrophoretic mobility shift assay (EMSA) and iNOS expression examined using immunohistochemical techniques in healthy nasal mucosa and chronically inflamed nasal polyps. Further NF-κB activity was assayed; by means of EMSA, in nasal epithelial cells isolated from both tissues.

NF-κB was activated in nasal polyps, but also to the same extent in healthy nasal mucosa. Uniform iNOS expression was localized within the airway epithelium in both inflamed and noninflamed tissues. Along with iNOS expression, concomitant NF-κB activation was found in nasal epithelial cells obtained from both tissues and no differences were observed when nasal mucosa and nasal polyp were compared.

These results suggest that constitutive nuclear factor-κB and concurrent inducible nitric oxide synthase expression in epithelial cells may play a physiological role in airway function.


The nasal epithelium represents the respiratory system’s first line of defence against inhaled stimuli such as viruses, bacteria, inflammatory stimuli and environmental pollutants. Airway epithelial cells are able to respond to such frequent environmental stresses by secreting pro-inflammatory mediators [1]. Nuclear factor-κB (NF-κB) plays a pivotal role in cellular responses to environmental changes by regulating the expression of specific sets of inducible genes involved in the immune response, and acute phase and inflammatory processes [2]. In resting cells, NF-κB resides in the cytoplasm as an inactive form complexed to a member of the family of inhibitory proteins (IκB) subunits [3]. Activation of NF-κB, in response to a variety of extracellular signals, leads to the release of the IκB subunit from the cytoplasmic complex, which is translocated into the nucleus where it activates its target genes upon binding to various κB motifs [4].

Active NF-κB is composed of two members of the Rel family of proteins. The best characterized form of this factor is the p50/p65 heterodimer; however, other members of the Rel family of proteins, including c-Rel, Rel B, v-Rel and P52, can form various hetero- and homodimers in a wide variety of cell types [5]. Previous studies have shown that NF-κB regulates the transcription of the inducible nitric oxide synthase (iNOS) gene in murine macrophages [6, 7], 3T3 fibroblasts [8] and human vascular smooth muscle cells [9]. Increasing evidence, based upon higher than normal levels of nitric oxide in the exhaled air of asthmatic patients [10, 11] and prominent immunostaining for iNOS in asthmatic airway epithelium [12], suggests that the iNOS isofrom may play a role in acute and chronic inflammatory airway diseases. In the upper airways, increased iNOS activity [13] and expression [14] have been shown in chronically inflamed nasal polyp tissues. Despite the reported iNOS upregulation under inflammatory conditions, exciting new findings have demonstrated that iNOS is continuously expressed in normal airway epithelium [15], implying a homeostatic role for this isofrom not previously considered. The mechanisms of the continuous and induced iNOS expression in epithelial cells are unknown. However, NF-κB activity in normal and inflammatory airway epithelium has not been fully examined.

It was hypothesized that NF-κB may be associated with airway epithelium iNOS expression, not only in airway inflammatory processes but also under physiological conditions. In the present study, this question was addressed by examining NF-κB activity, by means of electrophoretic mobility shift assay (EMSA), and iNOS expression, using immunohistochemical techniques, in healthy nasal mucosa and nasal polyps. In addition, NF-κB activity was assayed in nasal epithelial cells obtained from both tissues.
Material and methods

Subjects

Nasal polyps were obtained from 19 patients (12 males and 7 females; mean age 49±4 yrs) who underwent polypectomy because of nasal obstruction. Three of the patients had allergic rhinitis, eight had bronchial asthma and eight had no history of airway disease. Twelve patients were receiving topical nasal steroids, two of whom also received inhaled steroids, and five of whom also took systemic corticosteroids when symptoms required. Nasal mucosal tissues were obtained from 13 patients (nine males and four females; mean age 33±11 yrs) who required corrective surgical treatment for nasal obstruction. These patients had no clinical history of inflammatory airway disease and were not receiving any medication 1 month prior to surgery.

Tissue processing

Tissues were processed immediately following resection. For the NF-kB deoxyribonucleic acid (DNA)-binding activity assay, pieces of tissue were immediately frozen in liquid nitrogen and stored at -80°C. For the immunohistochemical techniques, the tissues were cut into several pieces of ~5-mm thick and fixed in periodate-lysine-paraformaldehyde, frozen in Tissue-Tek® and stored at -80°C. The explants were cut into several pieces of ~1-mm thickness and fixed in 10 mM N-acetylcysteine when symptoms required. Nasal mucosal tissues were obtained from 13 patients (nine males and four females; mean age 33±11 yrs) who required corrective surgical treatment for nasal obstruction. These patients had no clinical history of inflammatory airway disease and were not receiving any medication 1 month prior to surgery.

Epithelial cell culture

Nasal epithelial cells were cultured by means of the explant cell culture technique previously described [16]. Epithelial sections (one section per dish) were explanted into 35-mm diameter Falcon® Primaria™ plastic culture dishes (Becton Dickinson, Oxford, UK) containing 1 mL DMEM/Ham’s F12 1:1 and 100 U·mL⁻¹ penicillin, 100 µg·mL⁻¹ streptomycin, 0.25 µg·mL⁻¹ amphotericin B and 2% Ultroser G (Life Technologies). The explants were incubated in a 5% carbon dioxide/air atmosphere at 37°C. All explants were observed for cell outgrowth after 3 days and the medium was replaced every 48 h until the cells had grown to confluence, which normally took 3 weeks. The purity of the cultures and the identity of the cells were confirmed by light microscopy and immunofluorescence staining using an anti-pan cytokeratin monoclonal antibody (Sigma, St. Louis, MO, USA).

Nuclear extract preparation

Nuclear proteins were extracted from tissues according to a previously described method [17]. Nuclear proteins were extracted from epithelial cells as previously described [18], with a few modifications. The cell layers were washed twice with ice-cold Dulbecco’s phosphate-buffered saline (PBS) without calcium and magnesium (Gibco Laboratories, Paisley, UK) at pH 7.4, scraped off the dishes and sedimented by centrifugation 30 s (at 16,000 × g). The cell pellets were resuspended in 200 µL of ice cold hypotonic lysis buffer containing 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 2 µg·mL⁻¹ leupeptin and 5 µg·mL⁻¹ aprotinin. Cells were allowed to swell on ice for 15 min and then Nonidet P-40 (Boehringer Mannheim, Mannheim, Germany) was added to a final concentration of 0.6%. The tubes were gently agitated on a vortex mixer for 10 s to disrupt plasma membranes, and nuclei were sedimented by centrifugation for 30 s at 16,000 × g at 4°C. The nuclear pellet was resuspended in 35 mL of high-salt nuclear lysis buffer containing 20 mM HEPES (pH 7.9), 0.46 M NaCl, 1 mM ethylenediamine tetracetic acid (EDTA), 1 mM ethylene-glycol-bis-(β-aminooxyether)-N,N,N’,N’-tetracetic acid, 1 mM DTT, 0.5 mM PMSF, 2 µg·mL⁻¹ leupeptin and 5 µg·mL⁻¹ aprotinin and incubated for 15 min at 4°C with gentle orbital shaking. Nuclear proteins were extracted by centrifugation for 30 min at 16,000 × g at 4°C. Aliquots of the supernatant were immediately frozen and stored at -80°C. All steps were carried out at 4°C. Protein concentration was determined according to the method of BRADFORD [19].

Nuclear factor-kB electrophoretic mobility shift assay

The binding reactions were performed on ice in a volume of 15–20 µL containing 10 mM tris-hydroxymethylaminomethane HCl (pH 8.0), 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 5% glycerol, 5 µg·mL⁻¹ bovine serum albumin, 50 µg·mL⁻¹ poly (deoxyinosine-deoxycytidine) (Pharmacia Biotech, Uppsala, Sweden.), 100 µg·mL⁻¹ transfer ribonucleic acid (Sigma) and 4 µg of nuclear protein extract. A preincubation of 10 min was allowed prior to adding 20,000 counts per minute 32P-end-labelled double-stranded oligonucleotide (5'-AGTTGAGGGGACTTTCC-CAGGC-3') containing the consensus NF-kB site (Pro mega, Madison, WI, USA), and the incubation continued for a further 15 min period. The NF-kB complexes were separated on a 4% non-denaturing polyacrylamide gel (previously electrophoresed for 45 min at 5 V·cm⁻¹) in 0.3 x tris-borate-ethylenediamine tetracetic acid (TBE) buffer for 1.5 h at 10 V·cm⁻¹. The gel was transferred on to DE 81 cellulose paper (Whatman, Maidstone, UK), dried under vacuum at 80°C and exposed overnight at -70°C to an X-Omat AR film (Kodak, Rochester, NY, USA) using an intensifying screen. Autoradiographic bands were quantified by densitometric scanning. The specificity of the gel shift complexes was determined by preincubation with a 70-fold excess of unlabelled NF-kB probe or the nonspecific unlabelled activator protein (AP-1) oligonucleotide (Promega). In order to identify the subunits that constitute the NF-kB complex present in tissue and epithelial cell samples, specific antisera recognizing the P50, P65 and c-Rel subunits (generously provided by N. Rice) were preincubated with the nuclear extracts for 10 min prior to the addition of the labelled probe.

Inducible nitric oxide synthase immunostaining

Immunohistochemical staining for iNOS was performed using the immunoperoxidase technique. The thawed slides were rinsed in PBS (pH 7.4; Gibco) to remove OCT and incubated with 0.55% H₂O₂ in methanol in order to inactivate endogenous peroxidase. Sections were rinsed twice
for 5 min with PBS and incubated with human AB blood type sera (1:20 in PBS) for 20 min to block nonspecific reactions, then slides were incubated overnight at 4°C with a mouse monoclonal antibody raised against mouse macrophage iNOS, which recognizes human iNOS (Transduction Laboratories, Lexington, KY, USA) at 5 μg·mL⁻¹ in PBS. Mouse immunoglobulin (Ig)G2a myeloma proteins (Sigma) were used as negative control for the primary antibody. After two more 5-min washes, the slides were incubated at room temperature with biotinylated rabbit antimouse IgG (Dako, Glostrup, Denmark) diluted 1:400 in PBS containing human AB sera (1:200) for 30 min. After washing with PBS, the sections were incubated with a streptavidin/biotinylated horseradish peroxidase complex kit (Dako) for 40 min according to the manufacturer’s instructions. After washing, the sections were exposed to 0.05% 3,3'-diaminobenzidine (Sigma) and finally counterstained with Harris haematoylin solution (17 mg·mL⁻¹; Sigma), dehydrated and DPX- (BDH, Poole, UK) mounted. For the evaluation of immuno-histochemical iNOS protein signals, all tissue samples were coded and sections counted in a blinded fashion. For each tissue, a total of 20 fields were examined at a final magnification of ×400 and the number of positively-stained cells was recorded.

**Statistical analysis**

The NF-κB-binding band densities in tissues and epithelial cells are presented as mean±SEM. The unpaired t-test was used for comparisons between groups. A p-value of <0.05 was considered significant.

**Results**

**Nuclear factor-κB activity in nasal mucosa and nasal polyp tissue**

NF-κB-binding activity was measured by EMSA in nuclear extracts from healthy nasal mucosa and chronically inflamed nasal polyps. As shown in figure 1, both types of tissue contained one retarded band, which was completely absent in the presence of an excess of the unlabelled NF-κB probe, but not in the presence of an unrelated oligonucleotide (AP-I consensus site), confirming that the nuclear proteins bind specifically to the NF-κB consensus sequence in the EMSA. These data indicate the presence of constitutive NF-κB activity in human upper airway tissue, either normal or inflamed. When the intensity of the NF-κB-retarded bands was analysed by means of densitometry no difference was found between normal or inflamed tissues (fig. 1b). The NF-κB activity in polyps from patients who were receiving steroid therapy did not statistically differ from those who were not receiving treatment before polypectomy. Analysis of the subunit composition by means of supershift assay showed that specific antisera directed against the p50 subunit completely supershifted the NF-κB complex in both tissues (fig. 2), and the p65 antisera caused a partial further retardation of the specific NF-κB-retarded band. Anti c-Rel and nonimmune rabbit serum did not result in any supershift. These data show that both tissues assayed, healthy and inflamed, have the same pattern of subunit composition and reveal the predominance of the p50 subunit, as well as the presence of p65, in the activated NF-κB complexes detected in human upper airways.

**Inducible nitric oxide synthase immunoreactivity**

Since the expression of iNOS is normally associated with NF-κB activation, the presence of iNOS expression was examined, immunohistochemically, in the same healthy and inflamed nasal tissues exhibiting a high level of NF-κB DNA-binding activity. Both healthy nasal mucosa and nasal polyps showed prominent uniform iNOS staining of the whole epithelium (fig. 3a and c). The cytoplasm of all epithelial cells was labelled and no staining was seen in the nuclei. Additional staining was seen in the subepithelial glandular epithelium (fig. 4a), whereas vascular endothelium showed weak immunostaining (data not shown). Furthermore, a strong iNOS immunoreactivity was observed in the majority (>90%) of the inflammatory cells characteristically infiltrating the nasal polyp submucosa layer (fig. 4b). Control sections incubated with IgG2a were not stained at all (fig. 3b and d). These results indicate that the upper airway epithelium expresses the iNOS isoform independent of the inflammatory state of the tissue.
Nuclear factor-κB activity in nasal epithelial cells

To determine whether NF-κB is activated in nasal epithelial cells, EMSA was performed with nuclear proteins from cultured nasal mucosa and nasal poly epithelial cells. Two shifted bands were detected in both types of cell (fig. 5a). These shifted bands were eliminated by the addition of a 70-fold excess of unlabelled NF-κB probe. The addition of an excess of the unrelated AP-1 oligonucleotide did not alter the shifted band. These data demonstrate the specificity of the NF-κB/DNA complexes and reveal that nasal epithelial cells obtained from nasal mucosa and nasal polyps contain constitutively activated NF-κB in the nuclei. NF-κB band densitometry showed no difference between nasal epithelial cells obtained from healthy or inflamed tissues (fig. 5b). No shifted or a very faint band was detected in cultured fibroblasts obtained from these tissues (data not shown). Additionally, freshly isolated epithelial cells obtained by enzymatic dissociation were assayed by NF-κB activity and also contained similar levels of NF-κB-binding activity (data not shown). The supershift assays showed that p50 antisera completely supershifted the two NF-κB-retarded bands (fig. 6) both in nasal mucosa and nasal poly epithelial cells. In addition, the upper band was supershifted by p65 antisera and c-Rel resulted in a faint supershift. These data indicate that p50 is the predominant subunit in the activated NF-κB complexes and p65 is present in lesser amounts in the upper band. Whether the lower band is a p50 homodimer or a heterodimer of p50 with other subunits is yet to be elucidated.

Discussion

In this study, it was shown that NF-κB is activated not only in nuclei from chronically inflamed nasal polytissue, as expected, but also, to the same extent, in nuclei from healthy nasal mucosa. In addition, prominent and uniform iNOS immunostaining was observed within the airway epithelium, independent of the inflammatory state of the tissue. Along with iNOS expression, activated NF-κB was concomitantly also found in nonstimulated nasal epithelial cells isolated from both tissues and no difference in this was observed between nasal mucosa and nasal polyps. These data indicate that airway epithelial cells constitute a specific cell type containing both constitutively activated NF-κB and constitutively expressed iNOS.

The finding of constitutive NF-κB DNA-binding activity in airway epithelium is in contrast to previous in vitro studies which failed to find activated NF-κB in the nuclei of primary or immortalized human respiratory epithelial cells. These studies showed that exposure to oxidants, irritants and inflammatory signals known to be active in chronic airway disease leads to NF-κB activation and upregulation of some inducible genes [20–26], but, in the present work, NF-κB is activated without apparent stimulation. These discrepancies could be due to differences between nasal epithelial cells, which constitute the first line of defence of the airways, and epithelial cells from the lower respiratory tract, such as tracheal epithelial cells [22], bronchial cells [24] or epithelial cell lines [20, 21, 23]. In addition, no differences could be found between the NF-κB activity in epithelial cells from nasal polyps and that in nasal mucosa, suggesting that constitutive NF-κB activity is important for normal epithelial cell function. Consistent with the present findings, other authors failed to detect differences between the NF-κB activity in bronchial epithelial cells from normal subjects and that in asthmatic patients [27]. In contrast, other studies have reported strong NF-κB DNA-binding activity in freshly isolated bronchial epithelial cells from asthmatic patients, which was absent in control cells [28].

Given the key role of NF-κB in regulating genes involved in immune defence [2, 7] and taking into account the important function of the nasal mucosa and specifically of the epithelial cells in the defence of the lung against harmful agents, the present finding of constitutively activated NF-κB in airway epithelial cells, independent of the inflammatory state of the tissue, suggests that NF-κB may play a role in airway defence; however, the physiological relevance of this finding is yet to be elucidated.

Identification of the specific NF-κB/Rel proteins that constitute the activated complexes present in the nuclei of normal and inflamed tissues, as well as in nasal epithelial cells, may be an important step in determining their function. The present results show that normal and inflamed tissues have the same pattern of subunit composition, in which p50 is the predominant subunit in the activated NF-κB complexes and p65 is present in lesser amounts in the whole tissue. These results indicate that nuclei from whole tissue probably predominantly contain the p50 homodimer and a lesser amount of the p50/p65 heterodimer, although the presence of other members of the Rel family, such as p52 or Rel B, cannot be excluded. The supershift assays, performed in nasal epithelial cells, demonstrate that the nuclei of both nasal mucosa and nasal poly epithelial cells contain p50, p65 and c-Rel. The data indicate that the NF-κB constitutively activated complexes in nasal epithelial cells contain the p50 homodimer as well as the p50/p65 and the p50/c-Rel heterodimers. The presence of the c-Rel subunit only in epithelial cells and not in tissues may be related to the particular NF-κB/Rel composition of this cell type, which may be masked by other nonepithelial cells present in nasal mucosa and nasal polyps.
Fig. 3. – Immunohistochemical localization of inducible nitric oxide synthase (iNOS) in: a, b) nasal polyp; and c, d) nasal mucosa sections: a) iNOS immunoperoxidase staining of the nasal polyp epithelium; b) staining control in a nasal polyp section in which the iNOS antibody was substituted by immunoglobulin (Ig)G2a; iNOS immunoperoxidase staining of the nasal mucosa epithelium; and d) staining control in a nasal mucosa section in which the iNOS antibody was substituted by IgG2a. (Internal scale bars=50 μm.)
Previous studies have shown that heterodimers of p50/p65 and p50/c-Rel bind a variety of kB sites and cause transactivation by direct interaction with the basal transcription apparatus [29, 30], whereas the p50 homodimer lacks an identifiable transactivation domain and is unable, of itself, to significantly cause transactivation [5, 31].

The presence of constitutively activated NF-kB complexes in the nuclei of nasal epithelial cells may be related to the regulation of genes whose expression is continuously required for the normal function of the upper airway system. Since, in the present work, iNOS immunoreactivity was found in the same tissues, both healthy and inflamed, that contain NF-kB activated complexes, it is tempting to suggest that iNOS may be one gene continuously induced by NF-kB in the upper airway mucosa. Interestingly, the present results showed prominent iNOS immunoreactivity within the airway epithelium, both in nasal mucosa and chronically inflamed nasal polyps. In agreement with the present results, previous immunohistochemical studies have identified the presence of iNOS within airway epithelium, in normal nasal mucosa [32] as well as in human lung [33, 34]. In contrast to the present study, which failed to show differences between inflammatory and normal airway epithelium, other authors have reported iNOS immunoreactivity in the bronchial epithelium of asthmatics but not in control patients [12]. These discrepancies may be explained by the differences between upper and lower airway function. The presence of iNOS in upper airway epithelium suggests that NO derived from iNOS may participate in the defence of the respiratory system through its effects on neurotransmission, immune defence, cytotoxicity, ciliary beat frequency and mucus secretion [35].

In terms of iNOS localization, the main difference between healthy and inflamed tissues was found at the level of the inflammatory cells present in nasal polyps, which were strongly positive for iNOS, as expected, since nasal polyp...

Fig. 4. – Immunohistochemical localization of inducible nitric oxide synthase (iNOS) in nasal polyp sections: a) iNOS immunoperoxidase staining in subepithelial glandular epithelium; and b) iNOS immunoperoxidase staining in inflammatory cells infiltrated in the nasal polyp submucosa. (Internal scale bars=20 μm.)

Fig. 5. – a) Representative electrophoretic mobility shift assay showing specific nuclear factor-kB (NF-kB)-binding activity in cultured epithelial cells obtained from two nasal mucosa (lanes 1–6) and two nasal polyp tissues (lanes 7–12). Nuclear proteins (4 μg) were incubated with 20,000 counts per minute of 32P-labelled consensus oligonucleotide for NF-kB binding. The specificity of the gel shift complexes was determined by preincubation in the presence of a 70-fold excess of unlabelled NF-kB oligonucleotide (lanes 2, 5, 8 and 11) or excess noncompetitive unlabelled AP-1 oligonucleotide (lanes 3, 6, 9 and 12). The upper and lower band are indicated. The radioactivity at the bottom of the gel is free probe. b) Densitometric scanning measurement (mean±SEM) of the NF-kB-retarded bands corresponding to cultured epithelial cells obtained from nasal mucosa (n=7) and nasal polyp (n=13) tissues. AU: arbitrary units.
inflammatory cells produce a battery of cytokines, chemokines and growth factors [36], all of them well-established stimulators of iNOS in a variety of cell types [37]. In agreement with this iNOS localization, another histochemical study has demonstrated the presence of iNOS in inflammatory cells in the nasal mucosa of patients with chronic rhinitis [32]. The present results would support the concept that infiltrating cells are important in regulating NO production within inflammatory airways.

In summary, inducible nitric oxide synthase expression in normal and inflammatory upper airway epithelium as well as constitutive nuclear factor-κB activity in nasal epithelial cells from healthy nasal mucosa and nasal polyps have been demonstrated. The lack of differences, in both inducible nitric oxide synthase expression and the concurrent nuclear factor-κB activity, when nasal epithelium from healthy and inflamed tissues is compared would suggest a physiological role for these factors in the airways. Further studies, based on the analysis of inducible nitric oxide synthase promoter/protein interactions, will allow it to be established whether or not constitutive nuclear factor-κB activity is responsible for controlling the continuous inducible nitric oxide synthase expression in nasal epithelial cells.

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References


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Fig. 6. – Identification of the nuclear factor-κB (NF-κB)/ Rel components present in nasal mucosa epithelial cell (lanes 1–5) and nasal polyp epithelial cell (lanes 6–10) activated complexes. Supershift assays were performed with 4 μg of nuclear extracts, which were incubated with 20,000 counts per minute of 32P-labelled consensus oligonucleotide for NF-κB binding in the absence (lanes 1 and 6) or presence of antisera directed against p50 (lanes 2 and 7), p65 (lanes 3 and 8), κB (lanes 4 and 9) or normal rabbit serum (lanes 5 and 10). The radioactivity at the bottom of the gel is free probe.


