Nitric oxide (NO) has been identified as an important mediator in numerous physiological and inflammatory processes [1, 2]. NO formation was first described in endothelial cells [3], and was subsequently reported in many cell types, such as neurons, fibroblasts, platelets, macrophages, neutrophils and epithelial cells [4, 5]. NO is formed from l-arginine via the action of the enzyme NO synthase (NOS). NOS exists in constitutive (i.e. continuously present in the cells) and inducible forms (expressed following activation by cytokines and bacterial endotoxin). There are two different constitutive forms (cNOS), one typically found in neural cells [6] and another typically found in vascular endothelial cells [7], both of which are Ca2+/calmodulin-dependent. There is only one inducible form (iNOS), which is Ca2+-independent, found in activated cells, such as macrophages and hepatocytes [8].

Recent observations suggest that l-arginine-dependent NOS is present in different pulmonary cell types, and that it may play a role in various regulatory mechanisms in the airways and lung tissue [9]. The fact that inhaled NO is capable of producing selective pulmonary vasodilation in animals and humans [10] has provided a rational basis for its use in intensive care as an effective therapeutic agent in pulmonary hypertension [11], and the adult respiratory distress syndrome (ARDS) [12]. In addition to its vasorelaxant properties, there is evidence in vivo which suggests that NO plays a role in the modulation of bronchomotor tone in guinea-pigs [13], and humans [14]. Overall, these data suggest that NO may have relevant functions in the regulation of vascular and bronchial smooth muscle.

Added to its therapeutic potential, there is experimental evidence suggesting that NO may be an endogenous mediator in the pulmonary system. In this sense, NOS activity has been found in lung tissue preparations [15]. This activity has also been localized in several pulmonary cell types by histochemistry and immunohistochemistry [16], and NO has been found in parts per billion (ppb) levels in the air exhaled by experimental animals and humans [17]. Along these lines, an increased amount of NO in air exhaled by asthmatics has been reported [18]. This same report showed that the major proportion of the NO in the air exhaled by control subjects seemed to originate in the nasal airways; however, in mild asthmatics there was a major contribution from the lower respiratory tract.
airways [18]. The same authors have recently reported that the major source of NO from the upper airways is the maxillary sinus [19]. Nevertheless, a biochemical and histochemical analysis of NO production by the human upper airways, both in healthy and pathological conditions, has not been performed. Since nasal polyps constitute a chronic inflammatory process and NO plays an important role, both in acute and chronic inflammation [20, 21], we have examined the NOS activity levels in nasal polyps relative to those in healthy nasal mucosa. The purpose of this study was to ascertain whether NOS activity is present in the human upper airways, the proportion of isoforms, its precise location, and to determine the differences between inflamed and noninflamed tissues.

Material and methods

Tissues and reagents

Nasal polyps were obtained from nine patients (6 females and 3 males, aged 35–68 yrs) undergoing polypectomy; all patients were receiving topical corticosteroids and four of them received oral corticosteroid treatment as well. Nasal mucosa tissues were obtained from seven patients (3 females and 4 males, aged 32–61 yrs) operated for functional septoplasty; subjects did not receive any medication prior to surgery. The specimens (nasal mucosa and nasal polyps) were collected at the time of surgery in Hank’s balanced salt solution (HBSS) without calcium and magnesium (Gibco, Grand Island, NY, USA) containing 20 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), and were immediately processed. For NOS activity studies, tissues were frozen and stored at -70°C. For NADPH diaphorase histochemical staining, tissues were cut into several pieces at a thickness of about 5 mm and fixed in periodate-lysine-parafomaldehyde at 4°C for 3 h, and successively transferred to 0.1 M phosphate buffer, pH 7.4, 10% sucrose, 20% sucrose and 30% sucrose at 4°C for 3 h. The tissues were then frozen in Tissue-Tec ornithine carbamyl transferase (OCT) (Miles, Elkhart, IN, USA) in liquid nitrogen and stored at -70°C. Eight micron thick sections were obtained in a cryomicrotome, thaw-mounted on microscope slides coated with 2% 3-aminopropyltriethoxysilane (Sigma, St Louis, MO, USA). Slides were air-dried and stored at -40°C until assessment.

Assay of NOS activity

NOS activity was measured in nasal polyp and nasal mucosa homogenates, after partial purification by 2′,5′-adenosine diphosphate (ADP)-Sepharose chromatography of mucosa homogenates, after partial purification by 2′,5′-triethoxysilane (Sigma, St Louis, MO, USA). Slides on microscope slides coated with 2% 3-aminopropyltriethoxysilane were obtained in a cryomicrotome, thaw-mounted tissues were then frozen in Tissue-Tec ornithine carbamyl transferase (OCT) (Miles, Elkhart, IN, USA) in liquid nitrogen and stored at -70°C. Eight micron thick sections were obtained in a cryomicrotome, thaw-mounted on microscope slides coated with 2% 3-aminopropyltriethoxysilane (Sigma, St Louis, MO, USA). Slides were air-dried and stored at -40°C until assessment.

NADPH diaphorase staining

Sections were rinsed with phosphate-buffered saline (PBS) to remove the OCT compound. The slides were then incubated at room temperature with a mixture consisting of 0.25 mg·mL⁻¹ nitroblue tetrazolium, 1 mg·mL⁻¹ β-NADPH and 0.5% Triton X-100 in 0.1 M Tris buffer, pH 7.6 for 1 h, as described previously [16]. Sections were then washed in PBS and covered with Aquatex mounting medium (Merck, Darmstadt, Germany). For control purposes, either the β-NADPH was omitted or the 15 mM 2-nitrophenylphosphate was added, to inhibit endogenous phosphatases that can cause false-positive staining [23].

Statistical analysis

Within nasal polyp tissues, Wilcoxon matched-pairs signed-ranks test (two-tailed) was used to compare constitutive versus inducible NOS activity. The Mann-Whitney U-test was used to assess differences between constitutive NOS from nasal polyp and nasal mucosa tissues (Statistical Package for the Social Sciences (SPSS)/PC+ program; SPSS Inc., Chicago, Illinois, USA).

Results

Data from the citrulline release assay showed that NOS activity was present in normal human nasal mucosa and in nasal polyps. Total NOS activity ranged 1.29–18.0 pmol·min⁻¹·mg protein in nasal polyp tissues (mean±SD 5.94±5.71 pmol·min⁻¹·mg protein; n=9) and 0.01–0.57 pmol·min⁻¹·mg protein in nasal mucosa tissues (0.28±0.22 pmol·min⁻¹·mg protein; n=7). Whereas both Ca²⁺-dependent (constitutive) and Ca²⁺-independent (inducible) NOS activities were detected in nasal polyp tissues (fig. 1a), only Ca²⁺-dependent NOS activity was detected in healthy nasal mucosa tissues (fig. 1b). Constitutive NOS
activities in nasal mucosa and nasal polyp tissues ranged 0.01–0.57 pmol·min⁻¹·mg protein and 0.07–5.34 pmol·min⁻¹·mg protein, respectively, and were statistically higher in nasal polyps (1.27±1.60 pmol·min⁻¹·mg protein) than in nasal mucosa tissues (0.28±0.22 pmol·min⁻¹·mg protein; p<0.05, Mann-Whitney U-test).

In nasal mucosa 100% of total NOS activity was constitutive, whereas in nasal polyp 80% of the total NOS activity present was inducible. Inducible NOS activity in nasal polyps ranged 1.23–15.5 pmol·min⁻¹·mg protein, and this inducible form was statistically higher (4.67±4.57 pmol·min⁻¹·mg protein) than the constitutive form from the same polyp tissues (p<0.05, Wilcoxon matched-pairs test).

NADPH-diaphorase histochemical staining was strongly positive in all nasal polyp tissues studied, being localized mainly in airway epithelia (fig. 2a). Conversely, nasal mucosa tissues were faintly stained (fig. 2b and 2c), but NADPH-diaphorase activity was also localized in epithelia, either in the airway (fig. 2b) or in the glandular epithelia (fig. 2c).
No staining was found in control sections incubated without NADPH, and staining in the presence of ρ-nitrophenylphosphate was identical to that described above (data not shown), indicating that staining is due to NADPH-diaphorase activity and excluding a contribution from other enzymes.

Discussion

This study has shown the presence of NOS activity in inflamed and non-inflamed human upper airway tissues as detected either biochemically or histochemically. Our first approach consisted of measuring 3H-citrate production from 3H-arginine in tissue homogenates as an index of NOS activity. The high variability of NOS activity found in nasal polyp tissues may be due to individual differences, but the fact that patients were receiving topical and/or oral steroid treatment almost certainly influenced these results. For ethical reasons, it was not admissible to discontinue the treatment before surgery.

Direct evidence for the presence of NOS activity in upper airways has, thus far, not been reported in humans, despite a previous description of NOS activity in the mouse olfactory and vomeronasal system [24], by means of histochemistry, immunohistochemistry and in situ hybridization. In humans, functional studies, reported by Lundberg and co-workers [18], have described the presence of NO in exhaled air of control subjects, apparently originating in the nasal airways. Our results fit well with these data and confirm by means of biochemical assay that NOS activity is present in human upper airways. More interestingly, our data establish a clear relationship between inflammation and the amount and isoform of NOS activity. Our results indicate that the upper airways constitutively express a NOS, both in inflamed and non-inflamed tissues, but that in inflamed tissues, such as nasal polyps, inducible NOS is upregulated. These data suggest not only that NO may play a physiological role in the upper airways, but also that NO is associated to inflammatory processes in the airways. In this respect, our results agree with those describing an increase in the amount of NO in exhaled air from mild asthmatics, that seems to originate in the lower airways [18].

Histochemical staining of NADPH-diaphorase (nitroblue tetrazolium formazan formation) confirms the biochemical data (citrate production). NADPH-diaphorase is an enzyme which can be visualized by the reduction of tetrazolium salts to formazan in the presence of reduced NADPH. NADPH-diaphorase has been identified as an isoform of NOS [25]. Although NADPH-diaphorase is not an absolute marker for cells producing NO, the specificity of histochemical staining of NOS using NADPH-diaphorase reaction has been substantiated by some studies comparing histochemistry with immunostaining in intact tissues [26, 27]. Since the conversion of NADPH to NADP may be influenced by other enzymes, such as alkaline phosphatases or acid phosphatases, care must be taken to include appropriate controls and to avoid false positives [23]. For this reason, we have performed the staining either in the absence of β-NADPH or in the presence of ρ-nitrophenylphosphate, and the histochemical results presented here confirm the biochemical data.

Our results show that the staining is stronger in nasal polyp tissues (fig. 2a) than in nasal mucosa (fig. 2b and c), but in both tissues the staining is localized to the epithelia, either to the airway epithelia (fig. 2a and b) or to the glandular epithelia (fig. 2c). These data fully agree with the results of studies performed in human lung that localize NADPH-diaphorase throughout the airway epithelium [16] as well as in the NCI-H441 human bronchioalveolar epithelial cell line [28]. Thus, our findings suggest that the epithelium is a major source of NO production in human upper airways and stress the role of epithelial cells, not only as a structural cell, but also as a source of inflammatory mediators in chronically inflamed tissues, such as nasal polyps.

In summary, the present investigation shows that: 1) NOS activity is present in human upper airways; 2) it is localized in the epithelium; 3) constitutive NOS is expressed in both inflamed and noninflamed tissues; and 4) inducible NOS is only expressed in nasal polyp tissues. From these data, we conclude that NO may have an important physiopathological role in human upper airways.

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