



## Expression of nitric oxide synthases in leukocytes in nasal polyps

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### ABSTRACT

**Background:** Nitric oxide (NO) has various roles in airway physiology and pathophysiology. Monitoring exhaled NO levels is increasingly common to measure airways inflammation and inhaled NO studied for its therapeutic value in premature infants and adult respiratory distress syndrome. NO is produced by 3 isoforms of NO synthase (NOS1, 2, 3), and each can play distinct and perhaps overlapping roles in the airways. However, the distribution, regulation, and functions of NOS in various cells in the upper airways, particularly in leukocytes, are incompletely understood.

**Objective:** To characterize the expression of NOS isoforms in leukocytes in normal middle turbinate tissues (MT) and in inflammatory nasal tissue (nasal polyps, NP).

**Methods:** Normal MT tissue was collected from surgical specimens that were to be discarded. The NP samples were from surgical tissue archives of 15 patients with chronic rhinosinusitis. Isoforms of NOS in cells were identified by double immunostaining using NOS isoform-specific and leukocyte-specific (mast cell, eosinophil, macrophage, neutrophil, or T cell) antibodies.

**Results:** The proportion of total cells below the epithelium that were positive for each isoform of NOS was higher in NP than in MT. Each isoform of NOS was found in all leukocyte populations studied, and there were significant differences in the percentage of leukocytes expressing NOS isoforms between MT and NP.

**Conclusion:** All isoforms of NOS are expressed in leukocytes in MT and NP, and their expression varies among leukocyte types. Our data provide a basis to investigate the regulation, cell distribution, and distinct functions of NOS isoforms in normal and inflamed nasal tissues.

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### Introduction

In 2004, Ricciardolo and colleagues<sup>1</sup> provided a comprehensive review of the role of nitric oxide (NO) in airway physiology and pathophysiology and predicted that new developments in this field would have a marked impact on respiratory medicine. Indeed, monitoring exhaled NO levels has several clinical applications, and practice guidelines have been produced.<sup>2</sup> Inhaled NO has been studied in premature infants<sup>3</sup> and in adult conditions such as acute respiratory distress syndrome,<sup>4</sup> but additional studies are required. Advances in selective antagonism of nitric oxide synthases

(NOS)<sup>1,5,6</sup> or inhibition of a critical enzyme controlling its biosynthesis, namely, arginase,<sup>7</sup> promise to provide novel therapeutics in the future. However, NO has a diversity of functions that are mediated by a variety of its metabolites and can be protective against airway narrowing and injury or deleterious. NO is generated by 3 isoforms of NOS, NOS1 (neuronal), NOS2 (inducible), and NOS3 (endothelial). NOS1 and NOS3 are constitutively expressed, whereas NOS2 is expressed in activated cells and can be elevated in allergic and other inflammatory conditions. Interestingly, studies of NOS knockout mice have shown that NOS1 is involved in the pathogenesis of asthma (NOS1 knockouts are protected), whereas NOS2 and NOS3 knockout mice have increased susceptibility (NOS2 and NOS3 are protective).<sup>8</sup>

In the human airway, particularly the nasal mucosa, all 3 isoforms of NOS are found in epithelial cells.<sup>9–12</sup> NOS2 is also expressed in endothelial cells, glands, smooth muscle cells, and leukocytes in human and mouse nasal tissue.<sup>12–15</sup> In allergic rhinitis, the nasal mucosa has high expression of NOS2<sup>13,16</sup> in epithelial cells, and NOS2-derived NO may be involved in hypersecretion of

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**Table 1**  
Comparison of sources of normal middle turbinate (MT) and nasal polyp (NP) tissue

|             | MT                      | NP         |
|-------------|-------------------------|------------|
| Number      | 8                       | 15         |
| Female      | 4                       | 3          |
| Average age | 56.5 ± 6.4 <sup>a</sup> | 38.7 ± 4.9 |

<sup>a</sup>*P* < .05 compared with NP.

mucus, typical of rhinitis. However, no differences were found in NOS3 expression between allergic rhinitis subjects and controls.<sup>13,16</sup> Despite the differences for NOS2, data about levels of nasal or exhaled NO between allergic rhinitis subjects and normal controls<sup>17–22</sup> are inconsistent, and some of this inconsistency is associated with methodology for measuring nasal NO. Guida et al<sup>23</sup> demonstrated that important variables associated with elevated exhaled NO included asthma, respiratory symptoms without bronchial hyperresponsiveness, and nasal polyps (NP) in patients with chronic rhinosinusitis (CRS). Others have identified high levels of NOS2 expression and activity in NP,<sup>24,25</sup> although in another study, healthy subjects had significantly higher nasal NO concentrations than subjects with CRS.<sup>26</sup> This result seems contradictory to the high levels of NOS2 expression in CRS and reflects that our understanding of nasal NO synthesis and relationships to nasal and exhaled NO levels is incomplete. Furthermore, we have limited understanding of the distribution of NOS isoforms in normal nasal tissue and NP, and previous studies have focused on epithelial cells. Little is known about NOS expression in nasal inflammatory cells such as eosinophils, mast cells, macrophages, neutrophils, and T cells.

Accordingly, we investigated the expression of isoforms of NOS in various types of leukocytes in normal nasal tissue (middle turbinate [MT]) and in NP, an inflammatory tissue. We postulated that expression of isoforms of NOS in leukocytes would differ between normal and inflammatory states.

## Methods

### Human nasal tissues

All studies were approved by the Human Ethics Research Committee, University of Alberta. Informed consent for the use of tissues was obtained from each patient by a surgical release form signed before surgery, explaining that any tissue removed from the patient may be used for diagnosis, research, or disposal. This consent allowed access to the surgical pathology report, but not other

medical records such as the use of medications. Nasal polyps were obtained from endoscopic sinus surgery from patients with CRS at the University of Alberta Hospital, Canada, from archives of 2007 to 2009 (*n* = 15). Because access to normal nasal tissue is limited, we collected normal MT tissue from patients undergoing surgery for pituitary adenoma (*n* = 8) in 2010. All MT samples were from patients preselected to be nonallergic, based on their clinical history relating to rhinitis, seasonality, and perennial nature of any symptoms. Because the age distribution of patients undergoing surgery for pituitary adenoma and NP is significantly different in our institution, a significant difference was seen in average age between MT and NP groups (*P* < .05, Table 1). The proportion of males in NP (12/15) was not statistically different from that in MT (4/8) (*P* = .08).

### Tissue preparation for immunohistochemical staining

After excision, samples were placed in 10% neutral buffered formalin. After dehydration and paraffin embedding, 4-μm sections were generated from each tissue block.

### Immunohistochemical staining

The antibodies used to study NOS expression in leukocytes are summarized in Table 2. In addition, comparison of the target antibodies to isotype controls were done and isotypes did not show reactivity. Normal IgG from rat and mouse were obtained from Santa Cruz Biotechnology (Santa Cruz, California). Normal rabbit IgG was obtained from AbD Serotec (Raleigh, North Carolina). After antigen retrieval treatment (20 min at 90–95°C in citrate buffer, pH 6.0), deparaffinized sections were incubated with 4% hydrogen peroxide in methanol for 20 minutes to reduce endogenous peroxidase activity, and the sections were incubated in blocking solution (5% normal goat serum in phosphate-buffered saline [PBS]) for 30 minutes before incubation in primary antibody. Sections were incubated with anti-NOS antibody overnight at 4°C, washed 3 times with PBS, and incubated for 30 minutes with biotin-conjugated secondary antibody. Sections were then incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 1 hour at room temperature, and developed using the NovaRED peroxidase substrate kit (Vector Laboratories, Burlingame, California). Coverslips were placed on the slides with mounting medium (Cytoseal-XYL, Richard-Allan Scientific, Kalamazoo, Michigan).

The MC tryptase was stained with an alkaline phosphatase-conjugated anti-tryptase antibody and developed with the alkaline phosphatase substrate kit III (Vector Laboratories). We used monoclonal antibodies to stain leukocytes (Table 2), anti-ECP (EG2) for

**Table 2**  
List of antibodies used for detection of NOS and leukocyte types

| Specificity/primary antibody                               | Final concentration (μg/mL) | Company <sup>a</sup> | Secondary antibody              | Final conc. (μg/mL) | Company             |
|------------------------------------------------------------|-----------------------------|----------------------|---------------------------------|---------------------|---------------------|
| <b>Nitric oxide synthase</b>                               |                             |                      |                                 |                     |                     |
| Rabbit α-human NOS1 pAb (IgG)                              | 4                           | Santa Cruz           | Biotinylated goat α-rabbit IgG  | 3                   | Vector Laboratories |
| Rabbit α-human NOS2 pAb (IgG)                              | 4                           | Santa Cruz           | Biotinylated goat α-rabbit IgG  | 3                   | Vector Laboratories |
| Rabbit α-human NOS3 pAb (IgG)                              | 4                           | Santa Cruz           | Biotinylated goat α-rabbit IgG  | 3                   | Vector Laboratories |
| <b>Mast cell</b>                                           |                             |                      |                                 |                     |                     |
| AP-conjugated mouse α-human MC tryptase mAb                | 2                           | R&D Systems          | —                               | —                   | —                   |
| <b>Eosinophil</b>                                          |                             |                      |                                 |                     |                     |
| Mouse α-human ECP (EG2) mAb (IgG <sub>1κ</sub> )           | 5                           | Pharmacia            | Biotinylated goat α-mouse IgG   | 3                   | Vector Laboratories |
| <b>Macrophage</b>                                          |                             |                      |                                 |                     |                     |
| Mouse α-human CD68 mAb (IgG <sub>1κ</sub> )                | 3.6                         | Dako                 | Biotinylated goat α-mouse IgG   | 3                   | Vector Laboratories |
| <b>Neutrophil</b>                                          |                             |                      |                                 |                     |                     |
| Mouse α-human neutrophil elastase mAb (IgG <sub>1κ</sub> ) | 1.5                         | Dako                 | Biotinylated goat α-mouse IgG   | 3                   | Vector Laboratories |
| <b>T cell</b>                                              |                             |                      |                                 |                     |                     |
| Rat α-human CD3 mAb (IgG <sub>1κ</sub> )                   | 10                          | Serotec              | HRP-conjugated rabbit α-rat IgG | 13                  | Dako                |

Abbreviations: AP, alkaline phosphatase; ECP, eosinophil cationic protein; HRP, horseradish peroxidase; mAb, monoclonal antibody; MC, mast cell; pAb, polyclonal antibody.

<sup>a</sup>Santa Cruz Biotechnology (Santa Cruz, California); R&D Systems (Minneapolis, Minnesota); Pharmacia (Uppsala, Sweden); Dako (Carpinteria, California); AbD Serotec (Raleigh, North Carolina); and Vector Laboratories (Burlingame, California).

eosinophils, anti-CD68 for macrophages, anti-elastase for neutrophils, and anti-CD3 for T cells. When primary antibodies were not conjugated (eg, NOS2 and EG2 staining), biotin-conjugated secondary antibodies were used and developed with the ABC-AP kit or an HRP-conjugated streptavidin/NovaRED peroxidase substrate kit (Vector Laboratories).

#### Quantifications and statistical analysis

After immunohistochemical staining, cell types and NOS isoforms were counted using a microscope (magnification  $10 \times 40$ , high-powered field [HPF] =  $0.196 \text{ mm}^2$ ). Cells were counted from 3 randomly selected (unless otherwise noted in the figure legends) HPFs in the nasal tissue below the epithelium in both MT and NP samples, and distant from the edge of the section. Total cell numbers in a field were determined by counting nuclei. All slides were blinded before counting. Data were analyzed statistically using the nonparametric Mann-Whitney test for comparison between 2 groups and 1-way analysis of variance corrected with the Bonferroni multiple comparison test for comparison between 3 or more groups. Differences between groups were considered significant at  $P$  values of  $<.05$ .

#### Results

##### Leukocyte abundance in nasal tissue

Eosinophils represented  $1.5 \pm 0.1\%$  ( $2.3 \pm 0.3/\text{HPF}$ ) of the total cells in MT, whereas in NP eosinophil abundance increased significantly ( $P < .01$ ) to  $6.2 \pm 1.1\%$  ( $11.1 \pm 2.2/\text{HPF}$ ; Fig 1). Similarly, macrophages, neutrophils, and T cells were more abundant in NP than in MT, both in percentage ( $4.1 \pm 0.3\%$  vs  $3.0 \pm 0.3\%$ ,  $P < .05$ ;

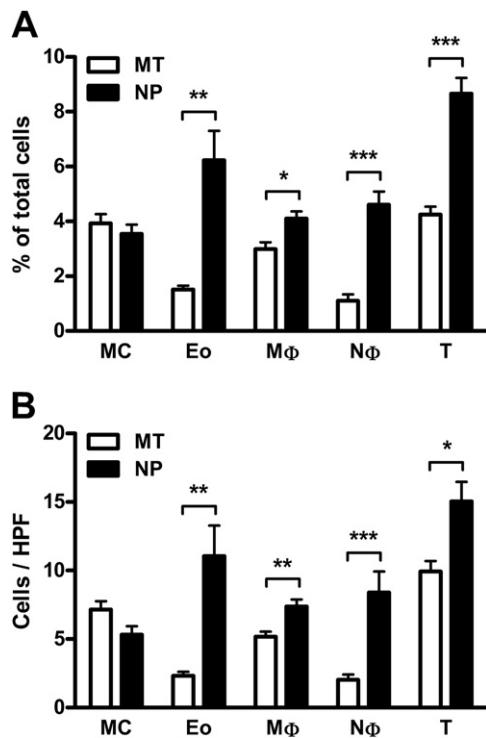


**Fig. 1.** Abundance of leukocytes in normal middle turbinate (MT) and nasal polyp (NP). Tissue sections from MT ( $n = 8$ ) and NP ( $n = 15$ ) were stained with leukocyte-specific antibodies for tryptase, eosinophil cationic protein (ECP), CD68, elastase, and CD3 for mast cells (MC), eosinophils (Eo), macrophages (MΦ), neutrophils (NΦ), and T cells (T), respectively. One to 3 high-powered fields (HPF) distant from the edge of the section from 3 slides for each sample (total, 7–9 HPF per sample) were randomly selected, and positively stained cells were counted and presented as a percentage of total cells in panel A, and the absolute number of cells per high-powered field in panel B (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ).

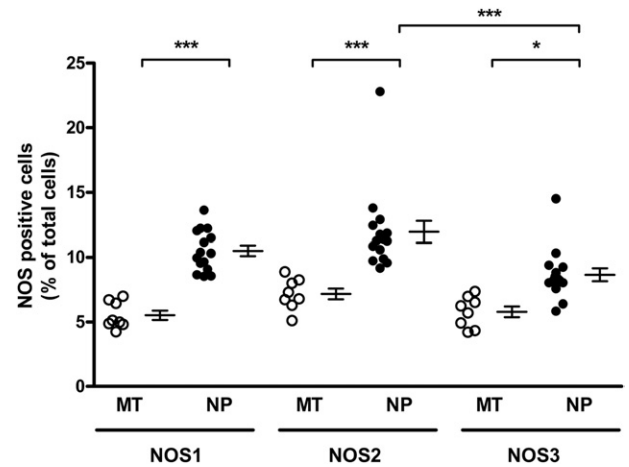


**Fig. 2.** Expression of NOS1, NOS2, and NOS3 in normal middle turbinate (MT) and nasal polyp (NP). Tissue sections from MT ( $n = 8$ ) and NP ( $n = 15$ ) were stained with nitric oxide synthase 1 (NOS1), NOS2, or NOS3 antibodies. One to 3 high-powered fields distant from the edge of the section from five slides (total, 11–15 fields) were randomly selected, and NOS isoform positive cells were counted. Data are presented as a percentage of total cells. (\* $P < .05$ , \*\*\* $P < .001$ ).

$4.6 \pm 0.5\%$  vs  $1.1 \pm 0.2\%$ ,  $P < .001$ ;  $8.7 \pm 0.6\%$  vs  $4.2 \pm 0.3\%$ ,  $P < .001$ ) and cell number ( $7.4 \pm 0.5$  vs  $5.2 \pm 0.3$ ,  $P < .01$ ;  $8.4 \pm 1.5$  vs  $2.0 \pm 0.4$ ,  $P < .001$ ;  $15.0 \pm 1.4$  vs  $9.9 \pm 0.8$ ,  $P < .05$ ) (Fig 1). However, no significant increase was seen in the percentage of mast cells ( $3.5 \pm 0.3\%$  vs  $3.9 \pm 0.3\%$ ) or their numbers ( $5.3 \pm 0.6$  vs  $7.2 \pm 0.6$ ) between NP and MT. When we examined mast cell phenotypes in nasal tissue, tryptase-positive mast cells were the dominant (>99%) phenotype in MT and, although we could detect chymase-positive mast cells or tryptase and chymase double-positive mast cells in NP, these phenotypes were infrequent (<1%; data not shown).

##### NOS expression in nasal tissue

NOS isoforms were detected in epithelial, endothelial, smooth muscle, and inflammatory cells, as reported previously.<sup>9–16</sup> We assessed NOS 1, 2, and 3 expression in the total cell population below the epithelium in MT and NP. An estimated  $5.5 \pm 0.4\%$  and  $10.5 \pm 0.4\%$  of total cells in MT and NP were NOS1 positive,  $7.2 \pm 0.4\%$  and  $11.9 \pm 0.8\%$  of total cells in MT and NP were NOS2 positive, and  $5.8 \pm 0.4\%$  and  $8.6 \pm 0.5\%$  of total cells in MT and NP were NOS3 positive, respectively (Fig 2). Significant differences were seen between MT and NP in NOS1 ( $P < .001$ ), NOS2 ( $P < .001$ ), and NOS3 ( $P < .05$ ) expression. The percentage of NOS3-positive cells in NP was significantly lower than that of NOS2-positive cells ( $P < .001$ ).

We identified types of leukocytes that were NOS positive using double-staining with both enzyme-based immunohistochemistry (Fig 3) and immunofluorescence-based confocal microscopy (eFig 1). For details of the immunofluorescence staining procedure see eMethods. The percentage of NOS1- and NOS3-positive cells that were mast cells in NP ( $12.5 \pm 3.0\%$ ,  $1.3 \pm 0.7\%$ ) was significantly lower than that in MT ( $35.7 \pm 8.0\%$  [ $P < .05$ ],  $20.7 \pm 2.7\%$  [ $P < .001$ ], Fig 4A, C). In contrast, the percentage of NOS2-positive mast cells in NP ( $66.9 \pm 3.9\%$ ) was significantly higher than that in MT ( $13.4 \pm 4.7\%$ ,  $P < .001$ , Fig 4B). NOS1-positive T cells in NP ( $21.0 \pm 2.7\%$ ) were significantly greater than in MT ( $8.6 \pm 2.7\%$ ,  $P < .01$ , Fig 4A). The percentage of NOS3-positive eosinophils ( $1.1 \pm 1.1\%$ ) in NP was significantly lower than in MT ( $23.3 \pm 6.2\%$ ,  $P < .01$ , Fig 4C). The percentage of NOS2-positive eosinophils in NP ( $39.7 \pm 6.2\%$ ) was significantly higher than in MT ( $15.6 \pm 4.0\%$ ,  $P < .05$ , Fig 4B). However, no statistical differences were seen between the percentages of NOS2-positive macrophages, neutrophils, and T cells in NP and MT (Fig 4B).

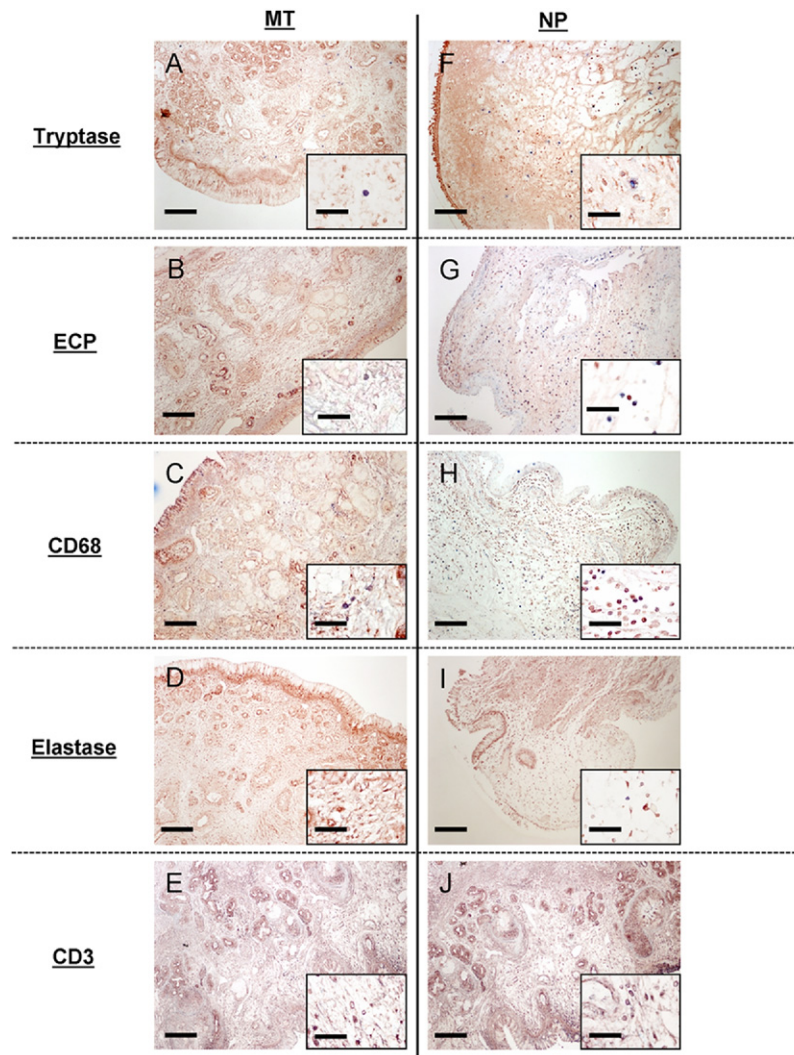


**Fig. 3.** Immunohistochemical staining for NOS2 and specific leukocyte markers in normal middle turbinate (MT) and nasal polyp (NP). MT and NP sections were double stained with both nitric oxide synthase 2 (NOS2) and leukocyte-specific antibodies. Tryptase, eosinophil cationic protein (ECP), CD68, elastase, and CD3 were used as markers of mast cells (MC), eosinophils (Eo), macrophages (MΦ), neutrophils (NΦ), and T cells (T), respectively. Inserts show the nature of cellular staining with several examples of single- and double-positive cells. In all panels except E, and J (for the T cell marker CD3), NOS2 staining is red, and the leukocyte marker is blue. In panels E and J, the staining is reversed. The scale bar in large pictures is 500  $\mu$ m and in inserts is 50  $\mu$ m.

## Discussion

We establish that the abundances of NOS1, 2, and 3 expression in all cell types (below the epithelium) are elevated in NP in comparison with normal MT (Fig 2). Moreover, for the first time, information is provided on the distribution and abundance of the expression of 3 NOS isoforms in various types of leukocytes in normal (MT) and inflamed (NP) human nasal tissue. Each isoform of NOS was found in all leukocyte types studied, and there were some significant differences in the percentage of leukocytes expressing NOS isoforms between MT and NP (Fig 4). Our results on NP (Fig 1) also concur with those of Igarashi et al,<sup>27</sup> who showed that eosinophil numbers are increased in allergic rhinitis, but mast cell numbers are not. The increases that we identified in macrophages, neutrophils, and T cells in NP were not seen, however, in allergic rhinitis.<sup>27</sup> We assessed eosinophil abundance in our NP samples and investigated the relationship with expression of isoforms of NOS in leukocytes but found no consistent association between eosinophil numbers and the distribution and abundance of NOS isoforms.

The proportion of mast cells, eosinophils, and macrophages that expressed NOS2 was greater than the proportion of neutrophils and

T cells (Fig 4B). In normal (MT) nasal tissue, more than 40% of macrophages expressed NOS2, whereas less than 20% of other leukocyte types were NOS2 positive. Macrophages are well known to express NOS2, and NOS2 expression is greatly enhanced in macrophages exposed to inflammatory cytokines.<sup>1</sup> Accordingly, in NP a high proportion of macrophages ( $56.1 \pm 4.3\%$ ), as well as mast cells ( $66.9 \pm 3.9\%$ ) and eosinophils ( $39.7 \pm 6.2\%$ ) expressed NOS2. NOS2 expression, albeit in a low proportion of cells, was found in T cells and neutrophils in nasal tissue, consistent with reports that some graft-infiltrating human T cells express NOS2<sup>28</sup> and that some activated neutrophils from patients with sepsis express high levels of NOS2 messenger RNA.<sup>29</sup> Thus, in some situations T cells and neutrophils can express NOS2 in human tissues, although these 2 leukocytes do not appear to be major sources of NOS2 in NP.

Several publications have identified high levels of NOS2 expression and activity in NP,<sup>24,25,30</sup> allergic rhinitis<sup>13,14,31</sup> and aspirin-sensitive patients with asthma,<sup>32</sup> but none has focused on NOS2 expression in various types of leukocytes. Conversely, a reduction of NOS2 expression has been reported in sinusitis.<sup>33,34</sup> Interestingly, in a model of allergic asthma, knockout of NOS2 is associated with enhanced numbers of inflammatory cells in airways after

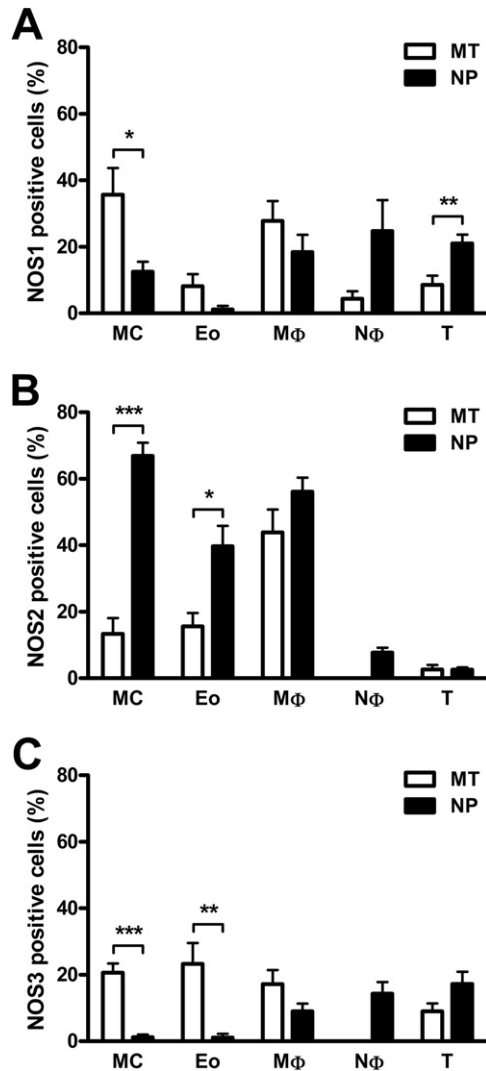


**Fig. 4.** Proportions of various cell types that are NOS1, NOS2, and NOS3 positive in normal middle turbinate (MT) and nasal polyp (NP). Tissue sections from MT ( $n = 8$ ) and NP ( $n = 15$ ) were double stained with a leukocyte-specific antibody and either NOS1, NOS2, or NOS3 antibodies in panels A, B, and C, respectively. Double positive cells were counted from a randomly selected total of 3 high-powered fields (HPF) distant from the edge except in B, where NOS2-positive eosinophils (Eo) and macrophages (MΦ) in NP were counted from a randomly selected total of 1 to 3 HPF, and NOS2-positive T cells (T) in NP were counted from 2 to 3 HPF. The NOS-positive cells in MT and in NP are presented as a percentage of each leukocyte (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ). MC, mast cell; NΦ, neutrophil; NOS, nitric oxide synthase.

allergen challenge, suggesting that NOS2 expression normally reduces the intensity of the inflammatory response.<sup>8,15</sup> Whether NOS2 expression in each type of leukocyte has a similar functional role in the respiratory tract remains to be determined.

Several cells in the upper airways can express NOS1 and NOS3, including parasympathetic neurons innervating nasal vessels, endothelial cells, and ciliated epithelial cells.<sup>11</sup> We found that expression of NOS1 in NP was higher than that in MT (Fig 2), but of its expression in leukocytes, the proportion of NOS1-positive cells was greater only for T cells (Fig 4). Interestingly, the proportion of mast cells expressing NOS1 was greater in normal MT tissue than in inflamed tissue, NP, in contrast to the marked increase of NOS2 in mast cells in NP. The major proportion of increased numbers of NOS1-positive cells in NP could be attributed to its expression in cells other than leukocytes. Shan et al<sup>35</sup> suggested that double immunofluorescence staining of tracheas for NOS1 and the nerve marker PGP 9.5 failed to demonstrate their co-localization, indicat-

ing that nerves may not be an important source of NOS1 in the murine airway wall. From a functional standpoint, Iijima et al<sup>36</sup> reported that NOS1 messenger RNA and protein were elevated in the airways by allergen challenge of sensitized mice and that in NOS1-deficient mice, allergen challenge induced less eosinophilia and a reduced up-regulation of NOS2 and NO production. Thus, NOS1 appears to be an important determinant of airway inflammation, and the regulation of its distribution and functions in various cells types requires additional study.

We also examined expression of NOS3 in leukocytes in human nasal tissue. Expression of NOS3 in NP was higher than that in MT (Fig 2), and of the leukocytes, a higher proportion of mast cells, eosinophils, and macrophages expressed NOS3 in NP than in MT (Fig 4). However, Kawamoto et al<sup>11</sup> reported no significant difference in NOS3 expression between normal nasal tissue and allergic rhinitis, although they did not detect NOS3 in inflammatory cells. NOS3 expression has been reported to be decreased after allergen challenge, and overexpression of NOS3 has a protective effect on allergic airway constriction in mice,<sup>37</sup> perhaps in part because NOS2, shown to reduce the intensity of inflammation in the airways, is dependent on NOS3 expression.<sup>15</sup>

The spectrum of observations in our study as well as in several others underlines that knowledge of the control of expression and the distinct functions of isoforms of NOS in different cell types in healthy and inflamed tissue microenvironments is limited. The potential relationship between our new information on expression of isoforms of NOS in leukocytes and the controversy about NO levels in nasal passages or in exhaled air must be viewed cautiously. Moreover, our data on NOS expression in different leukocytes may or may not be directly related to the pathogenesis of NP, as our study did not include analyses of medication use, duration of disease, or other variables. As shown by others, NOS2 expression in the epithelium is prominent and elevated in allergic rhinitis and may be an important source of nasal NO. NO is a short-lived gas, and it is rapidly converted by various chemical pathways into several products with various functional implications. For example, NO can nitrate tyrosine residues on proteins, nitrosylate thiol residues, or react with superoxide to produce peroxynitrate, with several downstream outcomes.<sup>1,38</sup> Much of the activity of leukocyte-derived NO is likely focused locally in the cell of origin or in cells in close proximity and thus is not a major source of nasal or exhaled NO. Further investigation is essential to more fully elucidate the significance of the multiple sources of NOS isoforms in the nasal mucosa.

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## Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi. 10.1016/j.anai.2011.12.013](https://doi.org/10.1016/j.anai.2011.12.013).

## eMethods

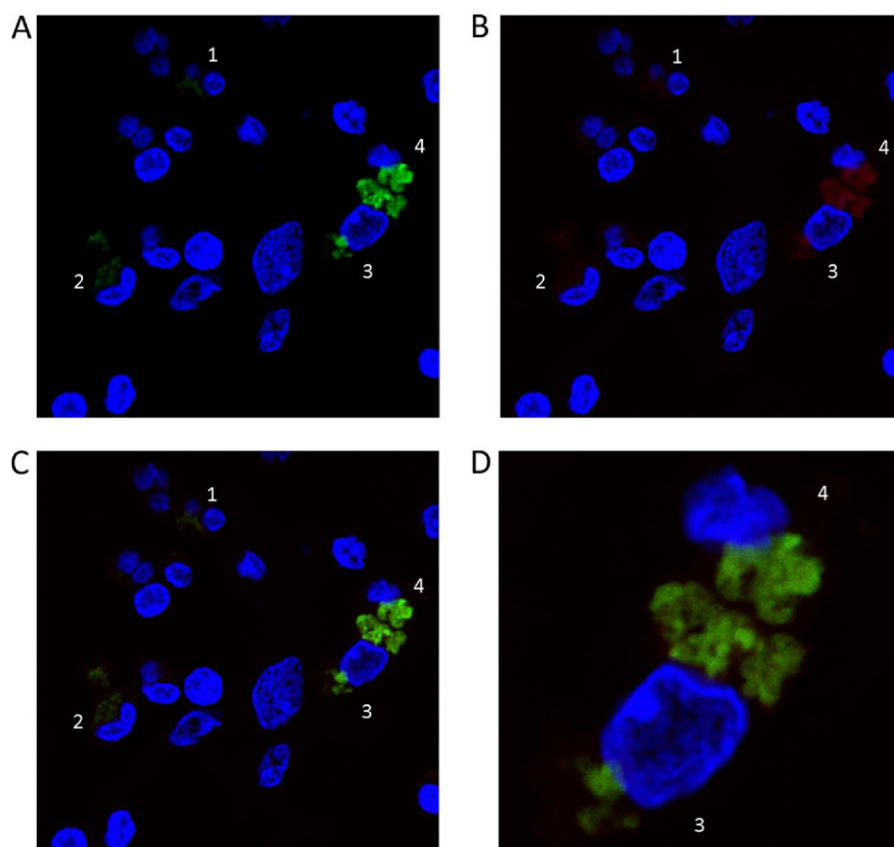
### *Sample collection and tissue preparation for immunofluorescence staining*

Tissues were collected and sections prepared as described in the manuscript.

### *Immunofluorescent staining*

Tissue sections were deparaffinized, and antigen retrieval treatment was done for 20 minutes at 90 to 95°C in citrate buffer, pH 6.0. Sections were incubated in blocking solution (5% normal goat serum in phosphate-buffered saline [PBS]) for 30 minutes before incubation in primary antibody. Sections were washed 3 times for 5 minutes with PBS, and then incubated with 5  $\mu$ g/mL rabbit  $\alpha$ -human NOS2 monoclonal antibody (Spring Bioscience, Pleasanton, California) at room temperature for 1 hour. Sections were washed 3 times for 5 minutes with PBS and then incubated with 1  $\mu$ g/mL goat  $\alpha$ -rabbit Alexa Fluor

594 labeled secondary antibody (Life Technologies, Grand Island, New York) for 30 minutes at room temperature. For tryptase staining, the Zenon mouse IgG labeling kit (Life Technologies) was used to directly label a mouse  $\alpha$ -human mast cell tryptase monoclonal antibody (R&D Systems, Minneapolis, Minnesota). Briefly, 1  $\mu$ g of tryptase antibody was combined with Alexa Fluor 488 labeled Zenon Fab fragments in a 6:1 molar ratio for 5 minutes. Tissue sections were incubated with the directly labeled tryptase antibody at a final concentration of 1  $\mu$ g/mL for 1 hour at room temperature. Sections were washed 3 times for 5 minutes with PBS, and coverslips were placed on the slides with SlowFade Gold mounting medium containing DAPI (Life Technologies). Images were obtained using an Olympus FV-1000 confocal microscope. Normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, California) and normal rabbit IgG (AbD Serotec, Raleigh, North Carolina) were used as isotype controls, and showed no fluorescence using identical settings on the confocal microscope as those shown in eFig 1.



**eFig. 1.** Immunofluorescent double-labeling of nasal polyp (NP) tissue for tryptase and nitric oxide synthase 2 (NOS2). Tissue sections from NP were double-stained with antibodies for both NOS2 and mast cell tryptase. NOS2 staining is shown in *red*, and tryptase staining is shown in *green*. Cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole), and are *blue*. *Panel A* shows tryptase signal only, and positively stained mast cells are labeled 1–4. *Panel B* shows NOS2 signal only in cells 3 and 4. *Panel C* shows merged signal of both tryptase and NOS2. *Panel D* is an enlargement of cells 3 and 4, showing NOS2-positive mast cells.