Signal transduction and oxidative processes in sinonasal polyposis

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Background: Nasal polyposis is characterized by impaired regulation of nasal tissue growth and is associated with chronic inflammation, sinus infections, and low levels of nitric oxide (NO). Based on its critical role in mediating cell growth and antimicrobial function, decrease of NO levels has been implicated in the pathogenesis of nasal polyposis.

Objective: We sought to evaluate mechanisms for the low NO level in polyposis, including factors regulating NO synthase (NOS) expression and activity and NO consumptive processes in nasal epithelial cells and nasal lavage fluid.

Methods: Eighteen patients with nasal polyposis and 8 healthy control subjects were studied. Nasal brushings, nasal lavage fluid, and nasal biopsy specimens were collected and analyzed.

Results: NO metabolite levels (nitrite and nitrate) in nasal lavage fluid from patients with polyps were less than those in control subjects, but activation of signal transduction and inducer of transcription 1, which regulates inducible NOS gene expression and protein expression, was present at higher levels in polyp than in healthy control tissue. Levels of arginine, methylarginine, and endogenous NOS inhibitors were similar between polyp and control tissue. In contrast, superoxide dismutase activity of polyp tissues was lower than that seen in control tissue and associated with increased nitrotyrosine, a biomarker of oxidant consumptive products of NO.

Conclusion: Taken together, these data suggest that the nasal polyp environment is characterized by abnormalities in NO metabolism that might predispose to altered regulation of tissue growth and infection.

Clinical implications: Identification of NO metabolic abnormalities might lead to novel treatments for sinonasal polyposis targeted against the pathways identified within this study. (J Allergy Clin Immunol 2007;120:1346-53.)

Key words: *Nasal polyposis, chronic sinusitis, nitric oxide, signal transducer and activator of transcription 1, superoxide dismutase, arginine, nitrite, nitrate*

Sinonasal polyposis (SNP) is pathologically defined by outgrowths of sinonasal mucosa into the paranasal sinuses and nasal cavity.[1] Chronic inflammation is a pervasive pathologic finding and is implicated as the common final pathway. SNP is commonly associated with chronic inflammatory airway processes; however, the initiating event or events in polyp formation remain largely unknown.[1] Notably, SNP is frequently characterized by microbial colonization in conjunction with intense eosinophil-dominated inflammation. It remains unclear whether bacterial colonization is an initial step or results from polyp-dominated outflow obstruction caused by the presence of 1 or more polyps.

In this context frequent infections and polyp formation dominate the course of sinonasal involvement in patients with cystic fibrosis (CF). Recent studies identify defects in the nitric oxide (NO) synthase signaling pathways in the airway epithelia of patients with CF that contribute to the susceptibility to frequent and more severe respiratory tract infections.[2] NO is an important regulator of immune surveillance, including antiviral and bactericidal effects,[2-4] but NO also inhibits cell proliferation,[5] DNA synthesis,[6,7] and collagen production. Prior studies have identified decreased nasal NO levels in patients with chronic rhinosinusitis (CRS) and SNP in general and in patients with CF, even before the onset of chronic respiratory tract infections.[8-10] Thus we reasoned that CRS with SNP might result from NO abnormalities in patients without CF, in part caused by the loss of NO antimicrobial effects, antiproliferative effects, or both.

Three types of NO synthases (NOSs), the enzymes responsible for endogenous NO production, have been described in human cells: inducible NOS (iNOS), which produces high levels of NO, and 2 constitutive NOSs that produce low levels of NO (endothelial NOS [eNOS] and neuronal NOS). Normal human respiratory epithelial cells *in vivo*, including sinonasal epithelium, produce NO through the continuous expression of iNOS.[11]

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Supported by grants from the National Institutes of Health (HL60917, HL69170, AI70649, and HL081064) and M01 RR018390 from the National Center for Research Resources and an internal grant through an institutional Research Programs Council grant.

Disclosure of potential conflict of interest: P. S. Batra is on the speakers' bureau for GlaxoSmithKline. S. C. Erzurum has received grant support from Asthmatix Alair. The rest of the authors have declared that they have no conflict of interest.

Received for publication February 2, 2007; revised July 20, 2007; accepted for publication July 24, 2007.

Available online October 12, 2007.

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0091-6749/$32.00

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doi:10.1016/j.jaci.2007.07.067

Abbreviations used
CF: Cystic fibrosis
CRS: Chronic rhinosinusitis
eNOS: Endothelial nitric oxide synthase
iNOS: Inducible nitric oxide synthase
NO: Nitric oxide
NOS: Nitric oxide synthase
SDMA: Symmetric dimethylarginine
SNP: Sinonasal polyposis
SOD: Superoxide dismutase
STAT1: Signal transducer and activator of transcription 1

Signal transducer and activator of transcription 1 (STAT1), a member of a family of proteins that transduce signals from cell-surface receptors to the nucleus and activate transcription by binding directly to regulatory DNA elements, is essential for host defense and for iNOS gene induction.[12] In this study we hypothesized that the low levels of NO in polyposis are due to abnormalities in NO metabolism within polyp tissue. To test this hypothesis, we investigated NOS expression, the molecular signal transduction factors that regulate NOS gene expression, the factors that regulate its enzymatic activity (including substrate arginine levels and endogenous methylarginine NOS inhibitors), and the oxidative NO-consumptive pathways by measure of superoxide dismutase (SOD) and nitrotyrosine, a marker of NO-oxidant reactions.

METHODS

Study population

Healthy control subjects and patients with untreated and treated SNP were recruited for this study in an institutional review board–approved protocol. Individuals were defined as having SNP based on the minimal criteria for CRS with nasal polyposis.[13] Both groups were screened for exclusion criteria: age less than 18 years, pregnancy, use of certain medications for at least 2 weeks (ie, topical nasal or systemic corticosteroids, antibiotics, leukotriene modifiers, topical or systemic antihistamines, mucolytics, H_2-blockers and/or proton pump inhibitors, topical/oral decongestants, topical nasal mast cell blockers, systemic herbal preparations, and vitamin C supplements), and history of allergy to the anesthetic agents used for the endoscopic biopsy. Healthy volunteers had no history of CRS, SNP, allergic rhinitis, or other inflammatory conditions of the paranasal sinuses and nose. Written informed consent was obtained from each patient before enrollment or an institutional review board exemption was obtained in the case of surgical specimen collection.

The Sinonasal Outcomes Test-20 (SNOT-20) survey and a screening questionnaire were administered to each prospectively collected subject.[14] Complete blood count with differential and a screening blood RAST against common environmental allergens were performed.

Nasal specimen collection

Nasal endoscopy with a 30° rigid endoscope (Karl Storz, Culver City, Calif) was performed to confirm the presence (or absence) of nasal pathology. Initially, the nares were physically cleansed of surface mucus. Subsequently, subjects had nasal lavage performed with a piston syringe containing 25 mL of Dulbecco's PBS (Gibco, Grand Island, NY). After lavage, brush samples and endoscopic biopsy specimens of the nares were collected. The lavage fluid was centrifuged, and the pellet containing mucus and cells was discarded. The cell-free lavage was analyzed in this study for nitrite, nitrate, and methylarginines. The brush and endobiopsy specimens provided the cells for Western analyses and SOD activity.

Endoscopically guided nasal brushing was performed with a 2 × 11–mm cytology brush (Bard Endoscopic Technologies, Billerica, Mass). Cells were immediately placed into bronchial epithelial growth medium prewarmed to 40°C (Cambrex, Walkersville, Md). The area of interest was also biopsied with endoscopic straight thru-cut biopsy forceps (Karl Storz).

Western analysis

Nasal brush cells were lysed in an ice-cold lysate buffer, separated by means of electrophoresis, and probed as previously described.[11,12] Primary antibodies included polyclonal STAT1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, Calif), phospho-STAT1 (1:1000; Cell Signaling, Danvers, Mass), glyceraldehyde-3-phosphate dehydrogenase (1:3200; Research Diagnostics, Inc, Flanders, NJ), iNOS (1:3000; Upstate, Lake Placid, NY), eNOS (1:1000; Affinity Bioreagents, Golden, Colo), neuronal nitric oxide synthase (1:1000, Affinity Bioreagents), monoclonal cytokeratin (1:2000; Dakocytomation, Carpinteria, Calif), and nitrotyrosine (1:3000, Upstate). After secondary antibody was conjugated to horseradish peroxidase, signal was detected by means of enhanced chemoluminescence (Amersham, Arlington Heights, Ill). Positive controls included A549 cells stimulated with IFN-γ or cytokine mixture containing IFN-γ, IL-1β, and TNF-α.[11]

Immunohistochemistry

Normal middle turbinate and nasal polyp tissues were fixed in 10% buffered formalin, paraffin embedded, and cut into 5-μm/L sequential sections. Primary antibodies included polyclonal anti-phospo-STAT1 (1:20) with antigen retrieval with sodium citrate buffer (pH 6.0), polyclonal anti-Von Willebrand factor (DAKO; 1:2000), and monoclonal anti-cytokeratin (AE1/AE3; 1:500) with antigen retrieval with protease treatment. Anti-AE1 immunoreacts with an antigenic determinant present on most subfamily A cytokeratins, and anti-AE3 reacts with antigenic determinant shared by subfamily B cytokeratins. Amino-ethyl-carbonyl chromogen color was detected with diaminobenzidine. Negative controls included secondary antibody alone.

Nitrite, nitrate, arginine, and other amino acids

As a measure of surface epithelial NO production, nitrite and nitrate concentrations in nasal lavage fluid were determined by using the ISO-NOP Nitric Oxide Sensor (World Precision Instruments, Sarasota, Fla), an amperometric sensor specific for NO, as previously described.[15]

Arginine and other amino acids were determined by means of stable isotope dilution HPLC with online electrospray ionization tandem mass spectrometry by using an API 365 triple quadrupole mass spectrometer (Applied Biosystems, Foster, Calif) with an Ionics EP10+ (Concord, Ontario, Canada) upgrade interfaced with a Cohesive HPLC (Franklin, Mass). $^{13}C_6$-Arginine was used as an internal standard, and samples were processed with mini DSC-SCX column (Discovery DSC-SCX SPE tubes; 1 mL; Supelco, Inc, Bellefonte, Pa) extraction after acidification with 0.2 N formic acid. Analytes were monitored in the multiple-reaction monitoring mode by using characteristic parent-daughter ion transitions for each analyte. A discontinuous gradient was used to resolve analytes comprised of

TABLE I. Demographic and clinical blood comparisons

Parameter	Healthy control subjects (n = 8)	Patients with nasal polyps (n = 18)	*P* value
Age (y)	35.5 ± 3.6	44.2 ± 2.9	.08
Male/female sex	3/5	13/5	.19
Race (white/black)	7/1	15/3	1
WBC (cells/mm^3)	5.6 ± 0.5	8.1 ± 0.6	.003
Eosinophil (%)	1.96 ± 0.4	8.43 ± 0.6	.005
Absolute eosinophils (cells/mm^3)	0.11 ± 0.03	0.66 ± 0.2	.005
Serum IgE (ng/mL)	11.4 ± 4.6	193.7 ± 75.3	.03
Atopy by mini-RAST	63%	80%	NA
Culture positive	0	10	NA
SNOT-20 score	0.13 ± 0.06	2.12 ± 0.5	.002

Values are presented as means ± SEMs.
WBC, White blood cell count in circulation; *Eosinophils*, percentage of circulating white blood cells; *Culture positive*, bacterial culture positivity, fungal culture positivity, or both in nasal secretion cultures; *SNOT-20*, Sinonasal Outcomes Score-20.

10 mmol/L ammonium formate aqueous solution (mobile phase A) versus methanol containing 0.1% formic acid and 5 mmol/L ammonium formate (mobile phase B). Control studies demonstrated that recovery of all analytes was greater than 85% with the methods used, with a coefficient of variance for each analyte of less than 15%.

SOD Activity

SOD activity was determined in nasal wash lavage fluid by using the rate of reduction of cytochrome c with 1 U of SOD activity defined as the amount of SOD required to inhibit the rate of cytochrome c reduction by 50%, as previously described.[16]

Statistics

Statistical comparisons were performed with JMP version 5.0 software (SAS, Cary, NC). Quantified variables were compared by using a Student *t* test of the SNP and healthy groups; a *P* value of less than .05 was considered significant. Summary statistics were compiled for clinical measures and are provided in Table I. For comparison of western OD (ImageJ Software; National Institutes of Health, Bethesda, Md), lanes were initially normalized to a control protein loaded equally on all gels and then normalized to cytokeratin or glyceraldehyde-3-phosphate dehydrogenase, resulting in a relative OD for each lane.

RESULTS

Study population

Four untreated and 14 treated patients with SNPs were enrolled (18 total), as were 8 healthy volunteers. The demographic and clinical characteristics of the populations are listed in Table I. The majority of patients with polyps were atopic (80%), whereas many healthy volunteers (63%) were also allergic to at least 1 allergen. Ten patients with SNPs also had asthma, and 2 of those were aspirin sensitive.

Western analyses for phospho-STAT1 and iNOS

Polyp lysates (n = 18) had higher levels of phospho-STAT1 than control epithelium (n = 8) (phospho-STAT1/cytokeratin: polyp tissue, 1.06 ± 0.18; healthy control tissue, 0.50 ± 0.083; *P* = .009). Similarly, iNOS expression was higher in polyp than control epithelium (iNOS/cytokeratin: polyp tissue, 2.23 ± 0.39; control tissue, 0.76 ± 0.17; *P* = .009; Fig 1). In contrast, eNOS expression was similar in control and polyp epithelial lysates (data not shown). STAT1 expression was comparable between groups (STAT1/cytokeratin: polyp tissue, 0.53 ± 0.071; control tissue, 0.353 ± 0.077; *P* = .102).

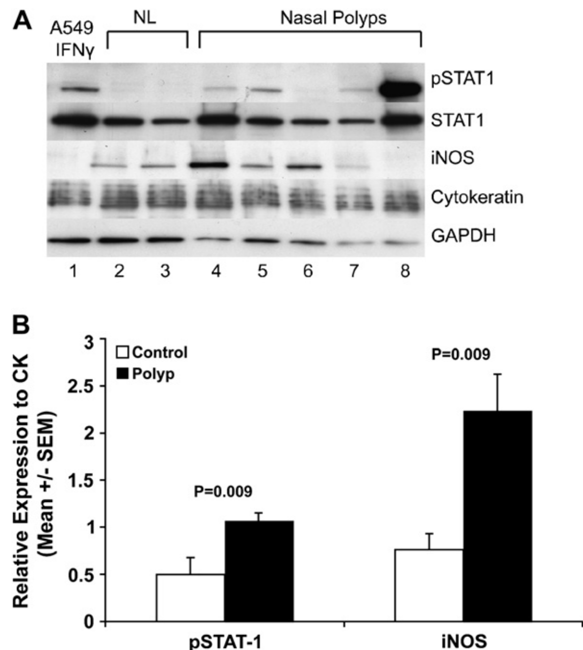

FIG 1. Increased phospho-STAT1 *(pSTAT1)* and iNOS expression in nasal polyps. **A**, Increased expression of phospho-STAT1 (91 kd) and iNOS (130 kd) in lysates of freshly obtained polyps compared with healthy nasal tissue (30 μg of protein per lane). Epithelial cells were stimulated with IFN-γ as a positive control. Cytokeratin confirms the epithelial nature of cell brushings, and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* confirms protein integrity. *NL*, Healthy tissue. **B**, Means ± SEMs. *CK*, Cytokeratin.

Positive immunoreactivity for phospho-STAT1 was seen in the nasal biopsy specimens of patients with nasal polyps but not in those of healthy subjects. The activation was striking in both epithelial and endothelial cells within polyps (n = 10, Fig 2). Nuclear localization confirmed phosphorylation, translocation to the nucleus, and thus activation of STAT1.

Nitrite and nitrate in nasal lavage fluid

NO production was assessed based on stable metabolites of NO in nasal lavage fluid, nitrite, and nitrate. Nitrite and nitrate levels in nasal lavage fluid from healthy volunteers (n = 7) and patients with nasal polyps (n = 8) revealed lower concentrations of nitrite and nitrate in patients compared with those in control subjects (mean ± SEM; nitrite: patients with polyps, nondetectable; control subjects, 0.62 ± 0.19 μmol/L; nitrate: patients with polyps, 8.85 ± 1.2 μmol/L; control subjects, 19.97 ± 1.91 μmol/L; all comparisons, *P* < .001; Fig 3).

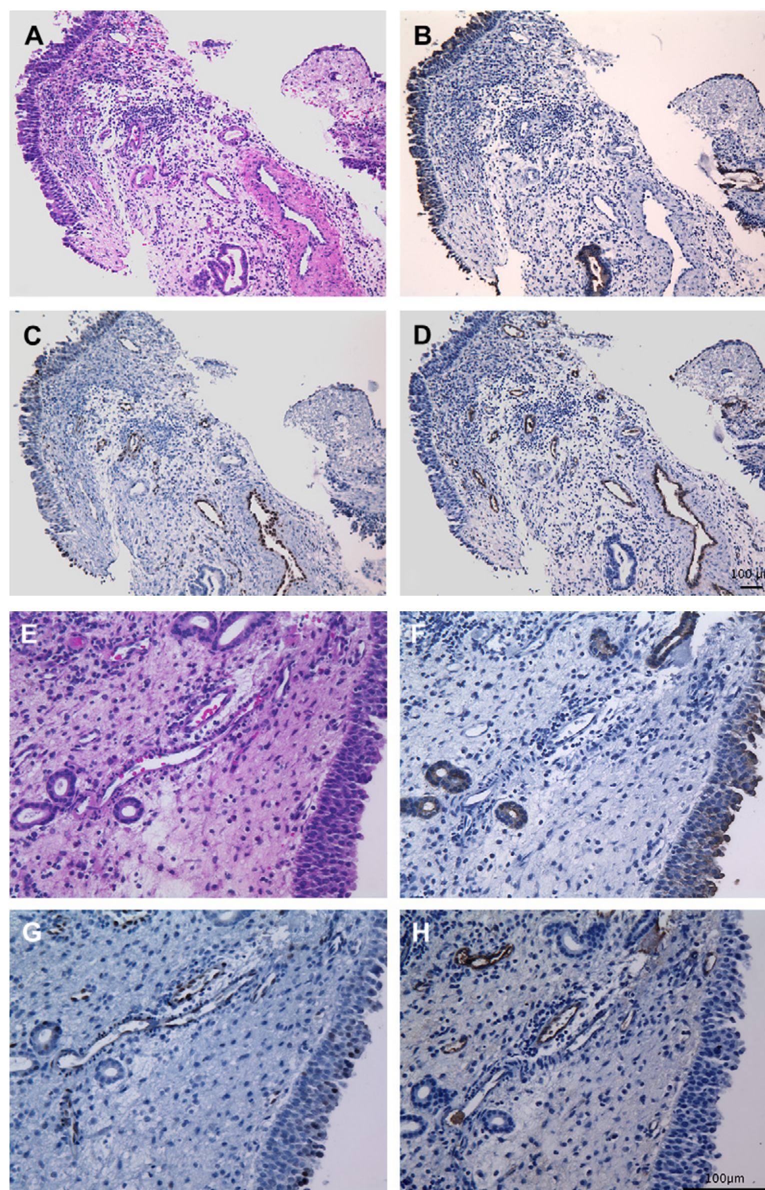

FIG 2. Nuclear localization of phospho-STAT1 in epithelial and endothelial cells. **A**, **E**, and **I**, Hematoxylin and eosin stain. **B**, **F**, and **J**, Immunostaining for cytokeratin to identify epithelial cells. **C**, **G**, and **K**, Phospho-STAT1. **D**, **H**, and **L**, Von Willebrand factor to identify endothelial cells.

Increased nitrotyrosine and decreased SOD levels in nasal polyps

Previous work suggested an oxidative nasal environment in CRS/SNP, as shown by an increase of oxidative modification of proteins in nasal biopsy specimens.[17] NO undergoes a direct bimolecular reaction with superoxide, yielding peroxynitrite, a reactive nitrogen species that leads to nonenzymatic nitration of tyrosines. Hence nitrotyrosine is a stable end product and biomarker of NO in oxidative environments. In fact, nasal polyp tissues had higher nitrotyrosine levels, as determined by means of Western analyses (nitrotyrosine/cytokeratin: patients with polyps, 0.86 ± 0.061; control subjects, 0.55 ± 0.04; $P = .0003$; Fig 4).

SOD enzyme function is essential to the clearance of superoxide in aerobic cells. In inflammatory airway diseases, such as asthma, SOD is inactivated in part by nitration and oxidation. The SOD activity in healthy control nasal epithelium from nasal brushings ($n = 6$) was greater than that from nasal polyp epithelium ($n = 15$; healthy control subjects, 49 ± 6 U/μg protein; patients with polyps, 29 ± 2 U/μg protein; $P = .02$; Fig 5). These data support antioxidant deficiency and oxidant excess in the nasal polyp tissue that leads to consumption of NO by reactive oxygen species.

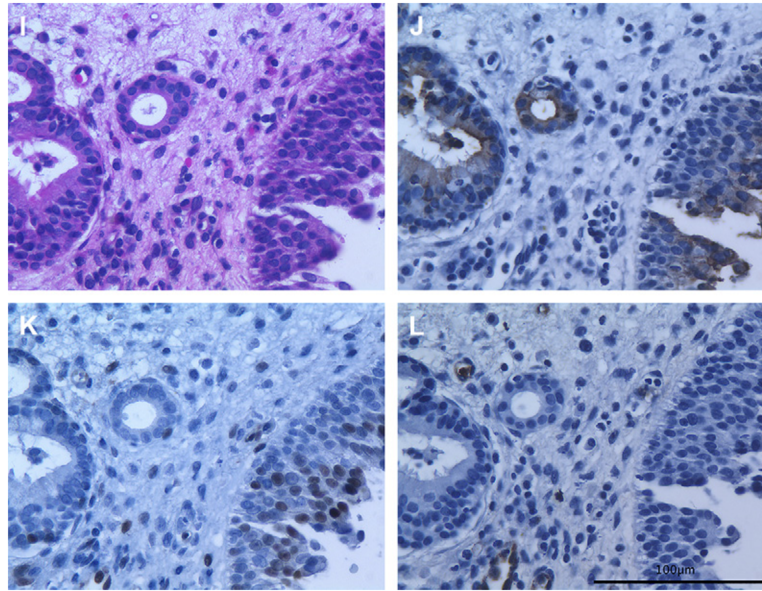

FIG 2. *(Continued)*

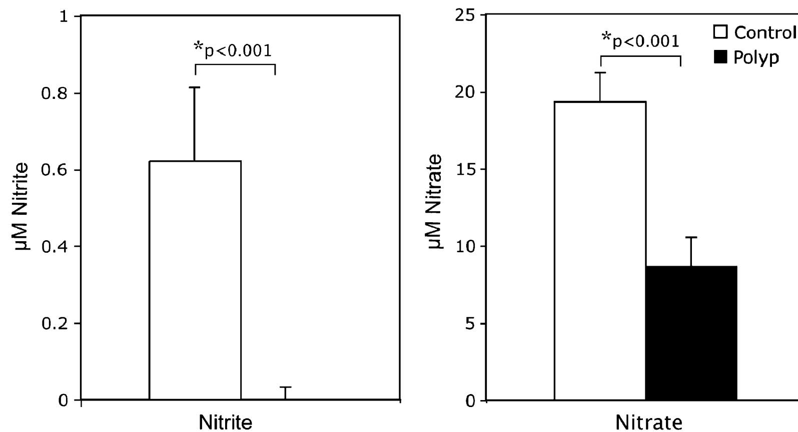

FIG 3. Nitrate and nitrite levels in nasal lavage fluid of 8 healthy control subjects and 7 patients with polyps. Means ± SEMs for nitrite and nitrate (in micromoles) are shown. *Significant.

Arginine, citrulline, and methylarginines in nasal lavage fluid

In addition to increased consumptive pathways, we investigated whether the low levels of nitrite and nitrate in polyp tissues are due to decreased enzymatic NO synthesis by evaluating the factors that regulate NOS activity, the endogenous NOS inhibitors asymmetric dimethylarginine and N^Gmonomethyl-L-arginine, symmetric dimethylarginine (SDMA), and substrate L-arginine levels.[15] Although SDMA does not directly inhibit iNOS, it is competitive with arginine for transport into the cell and thus might affect NO synthesis through effects on intracellular substrate availability. Quantification of arginine in nasal lavage fluid revealed that polyp and control samples had similar arginine levels (control subjects, 0.34 ± 0.091 μmol arginine; patients with polyps, 0.23 ± 0.023 μmol arginine; P = .39). The free methylarginines asymmetric dimethylarginine, N^Gmonomethyl-L-arginine, and SDMA were undetectable in nasal lavage specimens. Citrulline and ornithine levels were detectable in nasal lavage fluid, with a trend toward higher levels in the control group (mean ± SEM; citrulline: control subjects, 0.3 ± 0.1 μmol; patients with polyps, 0.10 ± 0.02 μmol; P = .09; ornithine: control subjects, 0.05 ± 0.02 μmol; patients with polyps, 0.015 ± 0.008 μmol; P = .07). The ratios of arginine/citrulline (control subjects, 1.51 ± 0.49; patients with polyps, 2.38 ± 0.29; P = .2) and arginine/ornithine (control subjects, 4.95 ± 1.14; patients with polyps, 3.91 ± 2.73; P = .7) were similar among control and polyp lavage fluid, suggesting that arginine metabolic pathways, including the arginase and NOS enzyme activities, were comparable among control and polyp tissues.

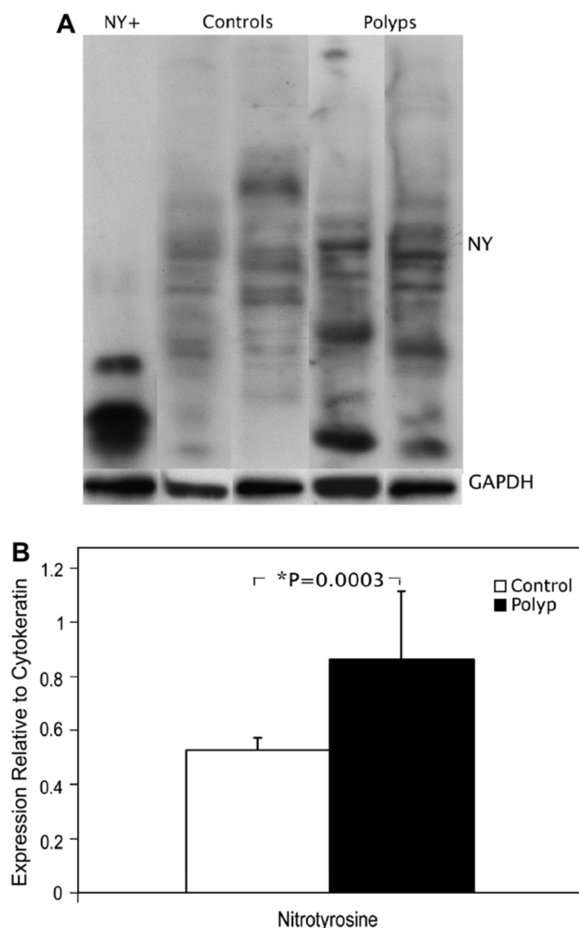

FIG 4. Nitrotyrosine *(NY)* levels in control and polyp epithelial cells. Lysates of freshly obtained nasal airway epithelial cells (30 μg of protein per lane) from healthy control subjects and patients with polyps are shown in panel **A**, along with a positive control of *in vitro* nitrated proteins *(lane NY+)*. **B**, Means ± SEMs of control and polyp tissue nitrotyrosine. *Significant.

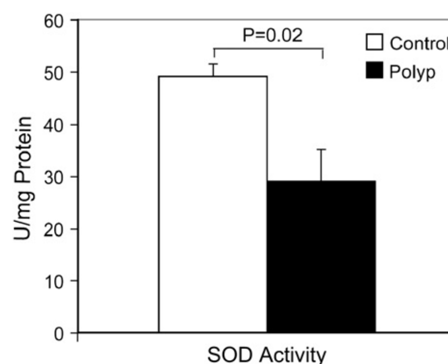

FIG 5. SOD activity in healthy control and polyp epithelium (in units per milligram of protein).

DISCUSSION

This study confirms and expands prior work that suggests iNOS expression and function are increased in patients with nasal polyps over that seen in healthy control subjects.[18-21] However, as previously shown in studies that assessed exhaled NO levels, nasal lavage data in the current study indicate that surface epithelial NO products are decreased in SNP.[12] Nasal NO levels are higher in the healthy volunteers, in whom concentrations reach between 20 and 200 ppb.[22] Significant decreases in values of nasal NO have been measured in specific disease states that are characterized by recurrent or chronic sinus infections, including SNP, acute rhinosinusitis, CRS, Kartagener syndrome, primary ciliary dyskinesia, and CF.[9,10,23-28] In contrast, this study demonstrated that iNOS was expressed at relatively abundant levels in nasal polyps. Among the known pathways for induction of iNOS, cytokine and double-stranded RNA-induced transcriptional control are well characterized.[22,29] IFN-γ induces phosphorylation of STAT1 (phospho-STAT1), its translocation to the nucleus of epithelial cells, and transduction of genes associated with inflammation, including iNOS.[30] Increased phosphorylation of STAT1 in this study mirrors data from asthma studies that report increased phospho-STAT1 over that seen in healthy control subjects.[22,29] This suggests that phospho-STAT1 is one mechanism that might be responsible for the induction of iNOS. The nuclear expression of phospho-STAT1 was confined to 2 cell types in polyps: epithelial cells and endothelial cells. This pattern is suggestive of an inflammatory process that is present at the interface of the nasal epithelium with the outside environment, as well as at the site of inflammatory cell recruitment (ie, endothelium in the vasculature). Studies implicate STAT1 activation in the cellular response to viral and chlamydia infections, and STAT1 activation might be a consequence of recurrent infections in the nasal polyp environment.[31,32] On the other hand, constitutive activation of STAT1 is an underlying mechanism leading to cytokine signaling and development of inflammation in asthma,[33] and given the strong association of sinus diseases and asthma, STAT1 activation in SNP might also be independent of external signaling events.

Despite the greater activation of signal pathways that induce iNOS and the greater iNOS protein levels, nitrite and nitrate levels in the nasal polyps were less than in control tissues. The lower levels of measured NO were not related to reduced substrate availability to the enzyme. Arginine levels were near equivalent in polyp and control tissue. Furthermore, the endogenously produced methylarginines that inhibit iNOS activity were undetectable in polyps. Cultures obtained from lavage specimens revealed no relationship between positive microbial cultures and either increased or decreased levels of nitrite and nitrate (data not shown). This suggests that bacterial enzymatic reduction of nitrate or nitrite is not a likely cause for low levels in nasal lavage fluid of patients with polyps. On the other hand, nitrotyrosine, a chemical modification that occurs during oxidative/nitrosative stress, was detected in nasal polyp tissues at greater than control levels.[18,21,32] NO and its metabolites interact with reactive oxygen species to generate nitrating agents that lead to 3-nitrotyrosine

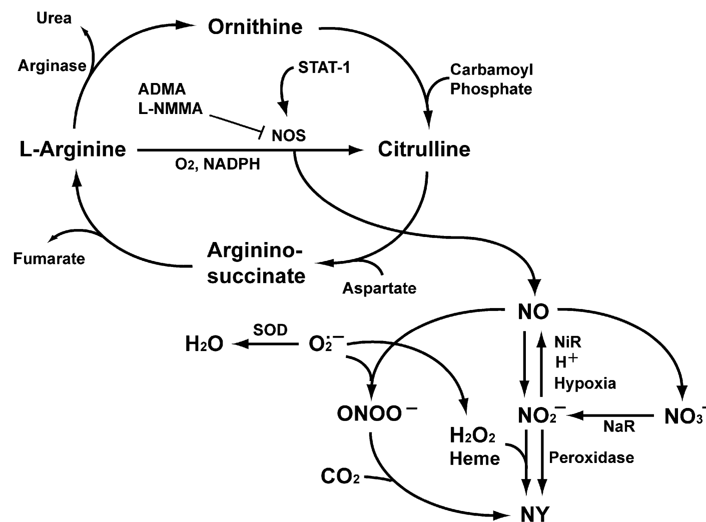

FIG 6. NO biochemical pathways. L-arginine is metabolized by urea cycle enzymes and through the enzyme iNOS, which produces NO. NO reacts with superoxide to form peroxynitrite, which ultimately generates nitrotyrosine *(NY)*. SOD, endogenous inhibitors of iNOS (asymmetric dimethylarginine *[ADMA]* and N^Gmono-methyl-L-arginine *[L-NMMA]*), and the bacterial enzymes nitrite reductase *(NiR)* and nitrate reductase *(NaR)* modulate NO levels in the sinonasal passages.

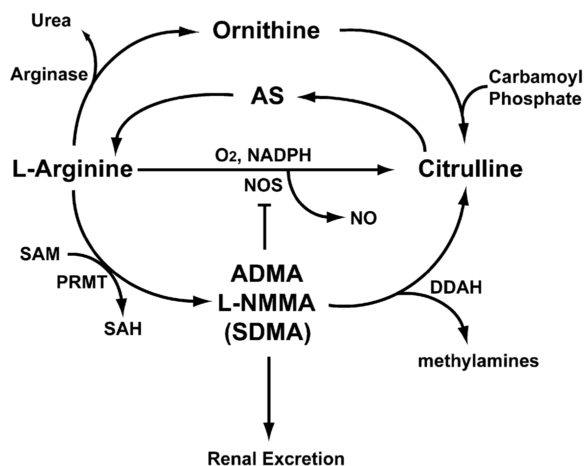

FIG 7. L-arginine metabolism. The substrate L-arginine is enzymatically converted to asymmetric dimethylarginine *(ADMA)*, N^Gmonomethyl-L-arginine *(L-NMMA)*, or symmetric dimethylarginine *(SDMA)* by protein arginine methylases *(PRMT)* in the presence of S-adenosylmethionine (forming S-adenylhomocysteine *[SAH]*). All methylarginines are excreted by the renal system or, in the case of ADMA and L-NMMA, enzymatically converted to methylamines by dimethylaminohydrolase *(DDAH)*, resulting in formation of citrulline. ADMA, L-NMMA, and SDMA reduce NO production.

formation in proteins.[32] Increases in tyrosine nitration have been observed in asthma, and the increase has been associated with loss of SOD activity.[34]

Previous studies showed that SOD activity was diminished in nasal polyps compared with that seen in control tissue, as well as within the plasma and erythrocytes of patients with polyps.[30,35] Although activity is reduced, expression levels of copper zinc SOD and extracellular SOD were greater in nasal polyps compared with those seen in control tissue.[36] These findings are analogous to SOD activity and expression in asthma in which SOD activity is decreased but SOD isoform expression tends to be higher than in healthy control subjects. The loss of specific activity has been attributed to oxidant inactivation of manganese SOD and CuZnSOD.[34,36,37] In this study SOD activity was decreased in polyp tissue compared with that seen in control tissue.

Collectively, these studies and previous work[19] provide evidence in favor of a role for reactive nitrogen and oxygen species in the pathogenesis of polyps. The STAT1 expression and activation and increased iNOS expression suggest the possibilities of an inciting external signaling event or constitutive activation of signal transduction driving the processes. However, the lack of increase of nitrite or nitrate levels, together with previous findings of low NO levels in patients with polyps, strongly supports an alteration of NO metabolism in the nasal tissues of polyps that might accelerate or lead to recurrence of polyps and lead to an environment more susceptible to microbial pathogens.

Graphic summaries of the pathways thought to be implicated in the pathogenesis of SNP are presented in Figs 6 and 7.

We thank J. Sharp and M. Baaklini for assistance in volunteer recruitment, L. Vargo and J. Drazba in the Lerner Research Institute Digital Imaging Core, and R. Prayson for nasal polyp tissues.

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