

Nitrosative stress in the bronchial mucosa of severe chronic obstructive pulmonary disease

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Background: Reactive nitrogen species, formed via the reaction of nitric oxide (NO) with superoxide anion and via (myelo)peroxidase-dependent oxidation of NO_2^- , have potent proinflammatory and oxidizing actions. Reactive nitrogen species formation and nitrosative stress are potentially involved in chronic obstructive pulmonary disease (COPD) pathogenesis. **Objectives:** To investigate the expression of markers of nitrosative stress, including nitrotyrosine (NT), inducible NO synthase (iNOS), endothelial NO synthase (eNOS), myeloperoxidase (MPO), and xanthine oxidase (XO) in bronchial biopsies and bronchoalveolar lavage from patients with mild to severe stable COPD compared with control groups (smokers with normal lung function and nonsmokers). **Methods:** The expression of NT, iNOS, eNOS, MPO and XO in the bronchial mucosa and bronchoalveolar lavage of patients was measured by using immunohistochemistry, Western blotting, and ELISA and correlated with the inflammatory cell profile. **Results:** Patients with severe COPD in stable phase had higher numbers of NT^+ and MPO^+ cells in their bronchial submucosa compared with mild/moderate COPD, smokers with normal lung function, and nonsmokers ($P < .01$). iNOS^+ and eNOS^+ but not XO^+ cells were significantly increased in smokers with COPD or normal lung function compared with nonsmokers ($P < .05$ and $P < .01$, respectively). In patients with COPD, the number of MPO^+ cells was significantly correlated with the number of neutrophils ($r = +0.61$; $P < .0025$) in the bronchial

submucosa. Furthermore, the number of NT^+ and MPO^+ cells was negatively correlated with postbronchodilator FEV_1 . **Conclusion:** These data suggest that nitrosative stress, mainly mediated by MPO and neutrophilic inflammation, may contribute to the pathogenesis of severe COPD. (J Allergy Clin Immunol 2005;116:1028-35.)

Key words: Bronchial biopsies, pathology, COPD

Chronic obstructive pulmonary disease (COPD) is a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases.¹ Patients with severe stable COPD have different inflammatory profiles in their bronchial mucosa compared with mild to moderate disease. Whereas T lymphocytes, particularly CD8^+ cells, and macrophages are the prevalent inflammatory cells in the bronchial mucosa of patients with mild to moderate COPD, total neutrophils predominate in severe COPD.² Reactive nitrogen species (nitrosants) have been implicated in the pathogenesis of COPD.³ In fact, cigarette smoking, the major cause of COPD, has a high concentration of nitric oxide (NO).⁴

Nitric oxide is also endogenously released in the bronchial mucosa, mainly by 2 functional isoforms of NO synthase: inducible NO synthase (iNOS) and endothelial NO synthase (eNOS).^{5,6} NO is a free radical able to interact with superoxide anion (O_2^-) to produce the formation of the strong nitrosant peroxynitrite (ONOO^-), which can lead to nitration (addition of $-\text{NO}_2$) of most classes of biological molecules. Peroxynitrite may thus provoke inhibition of mitochondrial respiration, protein dysfunction, and damage to cell membranes and DNA.⁷

Nitration is also possible via the activation of heme peroxidases, such as myeloperoxidase (MPO) and xanthine oxydase (XO), by H_2O_2 -promoting oxidation of NO_2^- . Furthermore, other oxidants that can oxidize NO_2^- to nitrating intermediates include HOCl, the product of MPO-catalyzed Cl^- oxidation.⁷ Exhaled NO is decreased in patients with severe stable COPD compared with mild to moderate disease and nonsmokers,⁸ suggesting that severe COPD may have a reduced NO production and/or increased NO consumption. In a previous study, an increased staining for the nitration marker nitrotyrosine (NT)- and for iNOS-positive cells was observed in

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TABLE I. Characteristics of subjects*

	N subjects	Age (y)	Sex (M/F)	PaO ₂ (mm Hg)	PaCO ₂ (mm Hg)	Smoking history (pack/y)	FEV ₁ (% predicted)	FEV ₁ /FVC (%)	FEV ₁ postβ ₂ (% from baseline)
Control nonsmokers	11	62 ± 12	5/6	80 ± 8	38 ± 3	0	115 ± 14	85 ± 10	—
Control smokers	13	60 ± 6	10/3	80 ± 11	39 ± 3	46 ± 29	106 ± 12	81 ± 6	—
Mild/moderate COPD	14	64 ± 7	11/3	75 ± 23	42 ± 12	35 ± 17	67 ± 13†§	60 ± 8†§	5 ± 1
Severe COPD	13	65 ± 10	12/1	66 ± 12	47 ± 7‡	49 ± 34	30 ± 8†§¶	42 ± 10†§¶	3 ± 1¶

*Data are expressed as means ± SDs.

†Significantly different from control smokers, $P < .0001$ and ‡ $P < .05$.

§Significantly different from control nonsmokers, $P < .0001$ and || $P < .05$.

¶Significantly different from mild/moderate COPD, $P < .0001$.

Abbreviations used

BAL: Bronchoalveolar lavage
COPD: Chronic obstructive pulmonary disease
eNOS: Endothelial nitric oxide synthase
FVC: Forced vital capacity
iNOS: Inducible nitric oxide synthase
MPO: Myeloperoxidase
NF-κB: Nuclear factor-κB
NO: Nitric oxide
NT: Nitrotyrosine
XO: Xanthine oxidase

induced sputum from patients with moderate stable COPD compared with nonsmokers, indicating that nitrosative stress may be exaggerated in the airways of patients with COPD.⁹

The aim of the current study was to investigate, by using immunohistochemistry, Western blotting, and ELISA, the expression of NT, iNOS, eNOS, MPO, and XO and to correlate their expression with the inflammatory cell profile in the bronchial mucosa and bronchoalveolar lavage (BAL) of patients with mild to severe stable COPD and in control smokers with normal lung function and nonsmokers.

METHODS

Subjects

We examined bronchial biopsies from 51 subjects by immunohistochemistry; 40 were current or exsmokers (quit for at least 1 year) with normal lung function ($n = 13$) or COPD ($n = 27$), and 11 were nonsmokers with normal lung function (Table I). The severity of COPD was staged by using the criteria of the Global Initiative for Chronic Obstructive Lung Disease¹: 14 smokers had mild to moderate COPD, 13 smokers had severe/very severe COPD, and 13 smokers had normal lung function (Table I). Six patients with mild to severe stable COPD and 5 smokers with normal lung function were used for Western blotting analysis. BAL was obtained from 7 healthy current smokers and 7 patients with mild to moderate COPD.

The study conformed to the Declaration of Helsinki, and informed consent was obtained from each subject. Bronchial biopsies and BAL were performed according to the local Ethics Committee Guidelines.

Lung function tests

Pulmonary function tests included measurements of FEV₁ and forced vital capacity (FVC) before and after the inhalation of 400 μg albuterol (6200 Autobox Pulmonary Function Laboratory; Sensor-medics Corp, Yorba Linda, Calif). The predicted normal values used were those from the European Community for Steel and Coal.¹⁰

Fiberoptic bronchoscopy, collection and processing of bronchial biopsies, and BAL

Fiberoptic bronchoscopy, bronchial biopsies¹¹ and BAL¹² were performed and processed¹¹⁻¹³ as previously described.

Immunohistochemistry

A panel of antibodies was used on cryostat sections to identify total T lymphocytes, CD4⁺, CD8⁺ T lymphocytes, macrophages (CD68⁺), neutrophils, NT⁺, iNOS⁺, eNOS⁺, MPO⁺, and XO⁺ cells. Primary antibodies were revealed as previously described.^{11,13}

Light-microscopic analysis was performed at a magnification of 630× for quantification of inflammatory cells and NT⁺, iNOS⁺, eNOS⁺, MPO⁺, and XO⁺ cells. All of the positive cells were quantified in the area 100 μm beneath the epithelial basement membrane with the use of a graded bar mounted at ocular level in several nonoverlapping high power fields until all of the available area was covered. The final result, expressed as the number of positive cells per square millimeter, was calculated as the average of all of the cellular counts performed in each bronchial biopsy. The immunostaining for iNOS, eNOS, MPO, and XO was also scored (range, 0 = absence of immunostaining to 3 = extensive immunostaining) in the complete (ie, both columnar and basal epithelial cells) bronchial epithelium. The final result was expressed as the average of all scored fields performed in each biopsy.

Western blot analysis for NT

Whole cell proteins were extracted and processed from bronchial biopsies as previously described.^{13,14}

Additional details on selection criteria of the patients, bronchoscopy, collection and processing of bronchial biopsies, immunohistochemistry, quantification of inflammatory cells, and Western blotting are provided in [Methods](#) in the Online Repository (see the online version of this article at www.jacionline.org).

ELISA

Free nitrotyrosine in BAL was measured by ELISA (#489540, NT tracer; and #489542, NT Enzyme Immune Assay antiserum) according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, Mich).

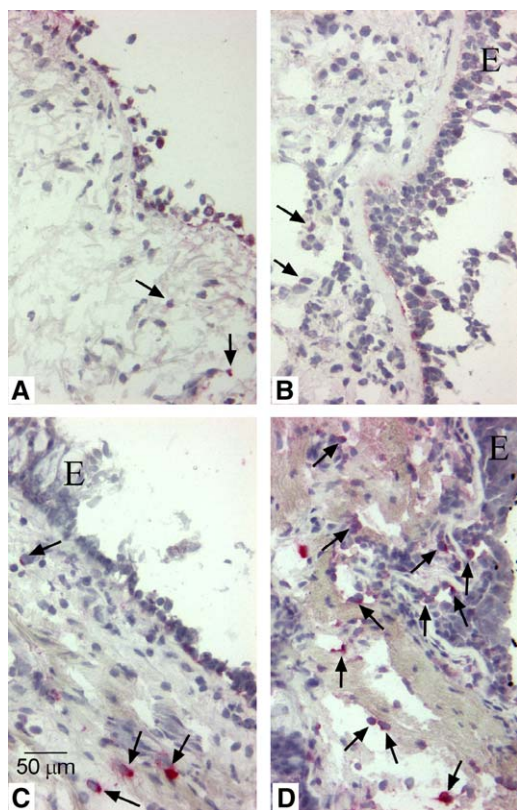


FIG 1. Photomicrographs showing the bronchial mucosa from a nonsmoker (**A**), a smoker with normal lung function (**B**), a patient with mild/moderate COPD (**C**), and a subject with severe COPD (**D**) immunostained for identification of NT⁺ cells (arrows) in the submucosa. Results are representative of those from 11 nonsmokers, 13 smokers with normal lung function, 14 patients with mild/moderate COPD, and 13 patients with severe COPD. E, Epithelium.

Data analysis

Group data were expressed as means $\pm$ SDs (Table I) for functional data or median (range) for morphologic data. Differences between groups were analyzed by using ANOVA for functional data. The ANOVA test was followed by the unpaired *t* test for comparison between groups. The Kruskal-Wallis test applied for morphologic data was followed, when significant, by the Mann-Whitney *U* test for comparison between groups. Correlation coefficients were calculated by using the Spearman rank method. Probability values of $P < .05$ were considered significant. Data analysis was performed by using the Stat View SE Graphics program (Abacus Concepts Inc, Berkeley, Calif).

RESULTS

Clinical findings

Subject characteristics are reported in Table I. The groups of subjects examined were similar with regard to age. Smoking history was similar in patients with mild/moderate or severe COPD and smokers with normal lung function. As expected from the selection criteria, the values of postbronchodilator FEV₁ (% predicted) and FEV₁/FVC (%) were significantly different in the groups

with mild/moderate and severe COPD compared with both smokers and nonsmokers. Patients with severe COPD also differed significantly from those with mild/moderate disease (for overall groups, ANOVA test, $P < .0001$ for postbronchodilator FEV₁% and FEV₁/FVC% values). PaO₂ was not significantly different in the 4 groups of patients examined. PaCO₂ in patients with severe COPD differed significantly from both control groups (ANOVA test, $P < .05$; Table I).

Immunohistochemical expression of NT in the bronchial submucosa

In the bronchial submucosa, NT immunopositivity was observed in inflammatory and structural cells, in the extracellular matrix, and in the true subepithelial basement membrane. The number of NT⁺ cells was higher in severe (200 [45-363]) compared with mild/moderate COPD (100 [21-232]; $P = .005$), smokers with normal lung function (56 [32-183]; $P = .0003$), and nonsmokers (64 [8-207]; $P = .003$; Fig 1). NT expression in mild/moderate COPD did not differ from either control group, nor did the 2 control groups differ significantly from each other.

Extracellular NT immunostaining score in the bronchial submucosa was higher in severe (2 [1-2.5]) compared with mild/moderate COPD (1 [0.5-2]; $P = .001$), smokers with normal lung function (0.5 [0.5-1.5]; $P < .0001$), and nonsmokers (1 [0.5-1.5]; $P = .004$). Extracellular NT immunostaining score was also higher in mild/moderate COPD compared with smokers with normal lung function ($P = .03$), but it did not differ in comparison with nonsmokers.

Western blotting analysis for NT

By using Western blot analysis, we found numerous protein bands immunoreactive for nitrotyrosine across a large range of molecular weights (<10 to >200 kd). The pattern of banding was different between COPD and smokers with normal lung function, particularly at the low molecular weight region. Bands ranging from 10 to 60 kd were more evident in patients with COPD than in smokers with normal lung function (see Fig E1 in the Online Repository in the online version of this article at www.jacionline.org).

ELISA for NT in the BAL

Free NT in BAL was significantly increased in patients with COPD (mean $\pm$ SE, 6.8 ± 0.4 ng/mL) compared with control smokers with normal lung function (5.3 ± 0.2 ng/mL; Mann-Whitney *U* test, $P < .01$).

Immunohistochemical expression of iNOS, eNOS, MPO, and XO in the bronchial submucosa

In the bronchial submucosa, the immunostaining for iNOS was mainly observed in the inflammatory cells. However, endothelial cells and fibroblasts/smooth muscle cells were also frequently positively stained. The number of total iNOS⁺ cells was significantly higher in patients with severe (56 [6-137]; $P = .003$) and mild/moderate

TABLE II. Quantification of NT, iNOS, eNOS, MPO, XO, and number of inflammatory cells in the bronchial epithelium and submucosa*

	Control nonsmokers	Control smokers	Mild/moderate COPD	Severe COPD	Kruskal-Wallis P value
Epithelium (score, 0-3)					
MPO	0.5 (0-1.5)	0.5 (0-3)	1.25 (0-2)	1.5 (0.5-3)	.14
iNOS	0.25 (0-1)	0.5 (0-2)	1 (0-1.5)	0.5 (0-2)	.44
eNOS	1 (0.5-2.5)	0.75 (0.5-1.5)	1.5 (0.5-2.5)	1 (0.5-2)	.32
XO	1.5 (0.5-2)	0.5 (0-2)	1 (0-2)	1 (0.5-2.5)	.19
Submucosa, cells/mm ²					
CD3	335 (47-523)	493 (198-1139)‡	590 (259-903)‡	430 (115-815)§	.01
CD4	168 (88-378)	235 (37-500)	249 (80-731)	219 (42-671)	.72
CD8	114 (15-286)	339 (78-657)‡	220 (86-523)‡	191 (59-355)‡	.01
CD68	284 (110-487)	520 (261-945)‡	663 (208-833)‡	549 (272-1054)‡	.002
Neutrophils	110 (59-270)	123 (17-308)	158 (78-512)	274 (47-500)†‡§	.02
MPO	129 (54-250)	105 (43-258)	158 (64-306)	242 (129-387)†‡§	.003
NT	64 (8-207)	56 (32-183)	100 (21-232)	200 (45-363)†‡§	.0008
iNOS	8 (0-40)	93 (16-125)‡	44 (8-100)‡	56 (6-137)‡	.001
eNOS	134 (43-193)	172 (77-303)‡	202 (64-277)‡	192 (89-280)‡	.04
XO	32 (0-169)	47 (0-188)	39 (0-148)	50 (8-297)	.94

*Results expressed as median (range).

†Mann-Whitney U test; significantly different from control smokers; ‡significantly different from control nonsmokers; §significantly different from mild/moderate COPD.

(44 [8-100]; $P = .009$) COPD and in smokers with normal lung function (93 [16-125]; $P = .001$) compared with nonsmokers (8 [0-40]). No significant differences were observed between subgroups of smokers (Table II; Fig 2).

In the bronchial submucosa, eNOS immunostaining was extensively observed in endothelial cells and occasionally in inflammatory cells. The number of total eNOS⁺ cells was higher in patients with severe (192 [89-280]; $P = .02$) and mild/moderate (202 [64-277]; $P = .01$) COPD and in smokers with normal lung function (172 [77-303]; $P = .02$) compared with non smokers (134 [43-193]). No significant differences were observed between subgroups of smokers (Table II; Fig 3).

In the bronchial submucosa, MPO was mainly expressed by neutrophils. A lower amount of this enzyme was also expressed by tissue macrophages. The number of total MPO⁺ cells was significantly higher in severe (242 [129-387]) compared with mild/moderate COPD (158 [64-306]; $P = .03$), smokers with normal lung function (105 [43-258]; $P = .001$), and nonsmokers (129 [54-250]; $P = .001$). Expression of MPO in patients with mild/moderate COPD did not differ significantly from either control group (Table II; Fig 4), nor did the 2 control groups differ significantly from each other.

In the bronchial submucosa, the immunoreactivity for XO was observed in the structural cells such as endothelial cells and smooth muscle. The number of total XO⁺ cells was not significantly different among the 4 groups of subjects studied (Table II).

Immunohistochemical expression of NT, iNOS, eNOS, MPO, and XO in the bronchial epithelium

Nitrotyrosine immunostaining was virtually absent in the bronchial epithelium of all subjects. In addition, no

significant differences were observed among the 4 groups of subjects studied for iNOS (Kruskal-Wallis, $P = .44$), eNOS (Kruskal-Wallis, $P = .32$), MPO (Kruskal-Wallis, $P = .14$), and XO (Kruskal-Wallis, $P = .19$) scored immunostaining in bronchial epithelial cells (Table II).

Immunohistochemical count of the inflammatory cells in the bronchial submucosa

The number of total T lymphocytes (CD3⁺ cells) was significantly higher in patients with mild/moderate COPD (590 [259-903] cells/mm²; $P = .006$) and in smokers with normal lung function (493 [198-1139]; $P = .01$) compared with nonsmokers (335 [47-523]). Scores in mild/moderate COPD also showed significantly increased values compared with severe COPD (430 [115-815]; $P = .05$). The number of CD3⁺ cells did not differ significantly between patients with mild/moderate COPD and smokers with normal lung function (Table II). The number of CD4⁺ cells was not different in the 4 groups of patients studied (Table II).

The number of CD8⁺ cells was significantly higher in mild/moderate (220 [86-523]; $P = .01$) and severe (191 [59-355]; $P = .05$) COPD and in smokers with normal lung function (339 [78-657]; $P = .01$) compared with nonsmokers (114 [15-286]). The number of CD8⁺ cells was not significantly different among the 3 subgroups of smokers with and without COPD (Table II).

Similarly, the number of CD68⁺ cells was increased in mild/moderate (663 [208-833]; $P = .001$) and severe (549 [272-1054]; $P = .001$) COPD and in smokers with normal lung function (520 [261-945]; $P = .008$) in comparison with nonsmokers (284 [110-487]). The number of CD68⁺ cells was not significantly different among the 3 subgroups of smokers with and without COPD (Table II).

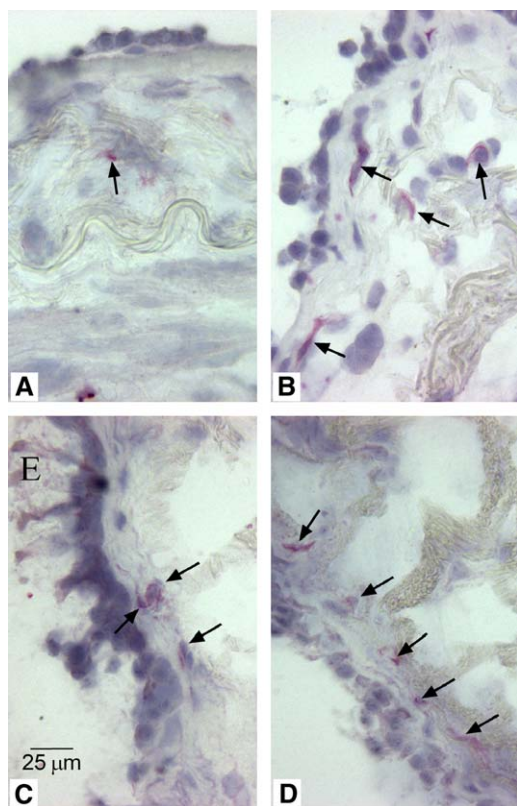


FIG 2. Photomicrographs showing the bronchial mucosa from a nonsmoker (**A**), a smoker with normal lung function (**B**), a patient with mild/moderate COPD (**C**), and a subject with severe COPD (**D**) immunostained for identification of iNOS⁺ cells (arrows) in the submucosa. Results are representative of those from 11 nonsmokers, 13 smokers, 14 patients with mild/moderate COPD, and 13 patients with severe COPD. E, Epithelium.

The number of neutrophils was significantly higher in severe COPD (274 [47-500]) compared with smokers with normal lung function (123 [17-308]; $P = .008$) and nonsmokers (110 [59-270]; $P = .009$). The number of neutrophils in mild/moderate COPD (158 [78-512]) did not differ significantly from severe COPD or either control group (Table II).

Clinical-pathological correlations

In patients with COPD, the number of NT⁺ cells in the bronchial submucosa was negatively and significantly correlated with the postbronchodilator FEV₁ (% predicted; $r = -0.59$; $P = .0025$; Fig 5, A) and postbronchodilator FEV₁/FVC ratio (%; $r = -0.51$; $P = .0086$; Fig 5, B). Similarly, in the same group of subjects, the number of MPO⁺ cells in the bronchial submucosa was negatively and significantly correlated with the postbronchodilator FEV₁ (% predicted; $r = -0.50$; $P = .011$; Fig 5, C) and postbronchodilator FEV₁/FVC ratio (%; $r = -0.49$; $P = .013$; Fig 5, D). Interestingly, the number of MPO⁺ cells in the submucosa was also positively correlated with the arterial partial pressure of CO₂ ($r = +0.73$; $P = .0009$).

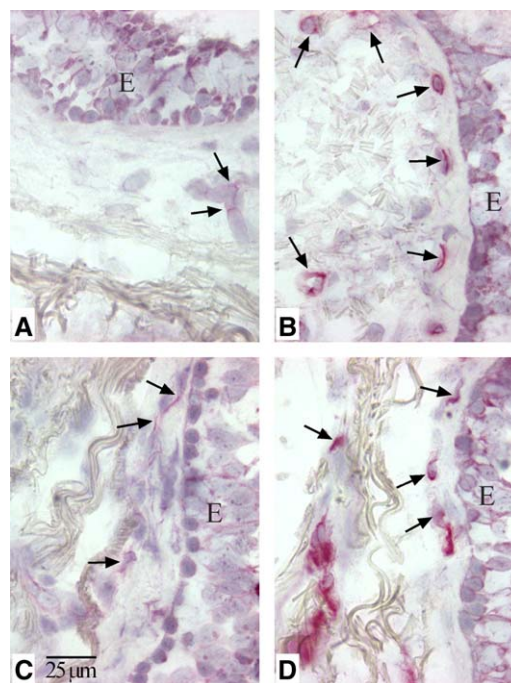


FIG 3. Photomicrographs showing the bronchial mucosa from a nonsmoker (**A**), a smoker with normal lung function (**B**), a patient with mild/moderate COPD (**C**), and a subject with severe COPD (**D**) immunostained for identification of eNOS⁺ cells (arrows) in the submucosa. Results are representative of those from 11 nonsmokers, 13 smokers with normal lung function, 14 patients with mild/moderate COPD, and 13 patients with severe COPD. E, Epithelium.

In all smokers ($n = 40$), the number of MPO⁺ cells in the submucosa was positively correlated with the number of neutrophils (neutrophil elastase-positive cells; $r = +0.64$; $P < .0001$). This association was maintained when the analysis was restricted to patients with COPD ($r = +0.61$; $P = .002$).

We did not observe any other significant correlations between morphologic and functional data or between morphologic parameters in the whole subject group or in subjects with COPD.

DISCUSSION

The current study demonstrated the presence of increased immunostaining for NT and an increased number of MPO⁺ cells and neutrophils in the bronchial submucosa of patients with severe stable COPD compared with mild/moderate COPD, smokers with normal lung function, and nonsmokers. Furthermore, we found a significant correlation between both NT⁺ and MPO⁺ cell numbers with the level of airflow obstruction in all patients with COPD, indicating that nitrosative stress may be involved in the progression of COPD. Finally, we observed an increased expression of iNOS and eNOS, but not of XO, in the bronchial submucosa of all smokers.

By using Western blotting analysis of bronchial biopsy protein extracts, we found evidence for tyrosine nitration of several proteins of various molecular weights (<10 tp >200 kd). Interestingly, there was a different pattern of banding between samples from patients with COPD and smokers with normal lung function. The identity and the functional role of these proteins in bronchial mucosa are still unknown and require a more detailed study using a proteomic approach to identify the many nitrated proteins.¹⁵

However, nitration may cause protein dysfunction; *in vitro*, nitration of histone deacetylase 2 causes loss of enzymic activity and is involved in the increased release of proinflammatory mediators (such as TNF- α and IL-8) from bronchial epithelial cells and alveolar macrophages and in the pathogenesis of corticosteroid resistance in smokers with COPD.^{16,17}

Elevated formation of nitrotyrosine has been detected in biopsies of different respiratory disorders, including acute respiratory distress syndrome,¹⁸ idiopathic pulmonary fibrosis,¹⁹ and asthma.^{20,21} The increased number of NT⁺ cells in the submucosa of bronchial biopsies in severe COPD also suggests the involvement of nitrosative stress in the pathogenesis of COPD, and in particular, in the progressive impairment of lung function. In fact, as in a previous study performed in patients with asthma,²¹ we found a correlation between the level of airflow obstruction and the number of NT⁺ cells. This suggests a potential role for tyrosine nitration in airflow obstruction via amplification of a nitrative/oxidative stress-mediated inflammatory process⁵ and degradation of extracellular matrix involving activated neutrophils and macrophages, resulting in alveolar wall destruction and small airways collapse.²² Our study is in line with, and extends to bronchial mucosa, a previous study by Ichinose et al⁹ performed in sputum cells, in which the number of inflammatory cells (macrophages and neutrophils) positive for nitrotyrosine was reported to be increased in patients with moderate COPD compared with nonsmoking control subjects.

Furthermore, we have found increased amounts of free NT in BAL from patients with mild to moderate COPD compared with smokers with normal lung function. This suggests that the 2 compartments, central and peripheral, of the airways are exposed to nitrosative stress in this disease.

Inducible NO synthase expression in human beings is regulated by both transcriptional and posttranscriptional mechanisms.²³ Transcriptional regulation involves the activation of the transcription factor nuclear factor (NF)- κ B. Recently, we have demonstrated that in the bronchial epithelium of smokers with COPD or normal lung function, there is an increased expression of p65, the major subunit of NF- κ B, compared with nonsmokers, and this in patients with COPD is related to the degree of airflow obstruction.¹³ This together with the current study suggests a potential role for NF- κ B in iNOS upregulation in the bronchial mucosa induced by tobacco smoking. It has also been shown recently that NF- κ B activation and iNOS

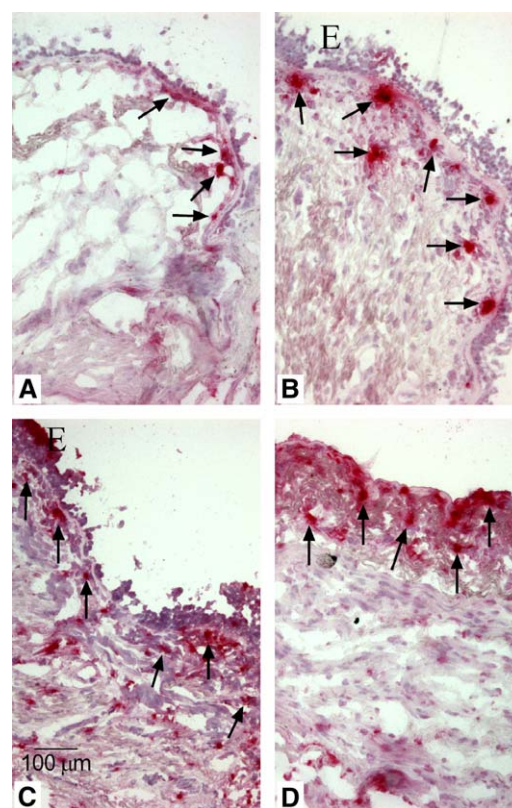


FIG 4. Photomicrographs showing the bronchial mucosa from a nonsmoker (A), a smoker with normal lung function (B), a patient with mild/moderate COPD (C), and a subject with severe COPD (D) immunostained for identification of MPO⁺ cells (arrows) in the submucosa. Results are representative of those from 11 nonsmokers, 13 smokers with normal lung function, 14 patients with mild/moderate COPD, and 13 patients with severe COPD. E, Epithelium.

induction occur in skeletal muscle of patients with COPD with low body weight.²⁴ All of these data suggest the involvement of iNOS in the smoking-induced airway inflammation in COPD.

Our study, for the first time, shows an increased expression of eNOS in the bronchial submucosa of smokers with normal lung function and COPD patients, suggesting a smoking-induced upregulation of eNOS. *In vitro*, oxidative stress may result in upregulation of eNOS expression, suggesting that oxidants in tobacco smoke may also provide an enhancement of eNOS expression.^{25,26} In addition, serum from smokers is capable of upregulating eNOS expression in human endothelial cells.²⁷ Interestingly, *in vitro*, eNOS has complex functions with production of both NO and superoxide anion.²⁸ All of these data suggest the involvement of eNOS in the smoking-induced airway inflammation in COPD.

The increase in both iNOS and eNOS expression suggests that the inflammatory process present in the airways of smokers may modulate NO production and that the different chemical fate of NO itself may depend on the airway microenvironment.²⁹ In particular, the over-

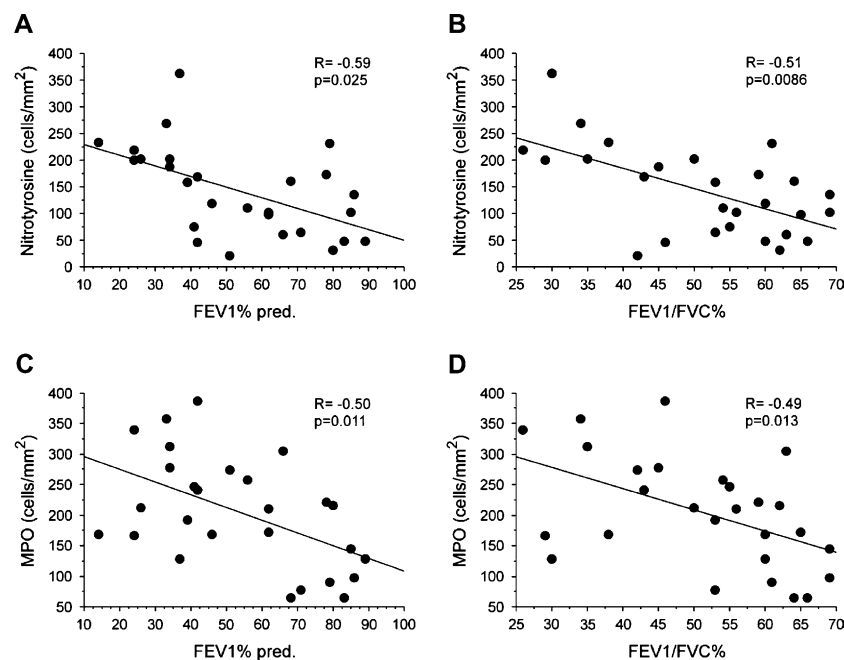


FIG 5. Regression analysis between postbronchodilator FEV₁ % predicted (**A and C**), FEV₁/FVC % (**B and D**), and numbers of NT⁺ (**A and B**) and MPO⁺ (**C and D**) cells in the submucosa of all patients with COPD. Correlation coefficients were calculated by using the Spearman rank method.

lapping release of reactive oxygen species facilitates the process of NO consumption via the formation of reactive nitrogen species and the subsequent generation of nitration reactions.

NO₂⁻ is a major end product of NO metabolism, and increased NO₂⁻ levels can often be detected in situations in which NO production is elevated. NO₂⁻ can be oxidized by heme peroxidases, such as myeloperoxidase, in the presence of H₂O₂, and by HOCl, a product of MPO-catalyzed Cl⁻ oxidation, resulting in the formation of reactive nitrogen intermediates capable of nitrating different tyrosine residues in many diverse proteins.³⁰ In this study, we also found a marked increase in MPO⁺ cells in the bronchial submucosa from patients with severe COPD, suggesting that MPO-dependent NT formation could be involved in the pathogenesis of COPD. We showed that in patients with COPD, MPO⁺ cells correlated significantly with neutrophils but not with macrophages, suggesting a major role for neutrophils in mediating nitrosative stress in the bronchial mucosa.³¹ This study also confirms our previous evidence of an increased number of neutrophils associated with severe COPD.¹¹ We did not find a significant correlation between NT⁺ cells and neutrophils or MPO⁺ cells. However, this discrepancy does not rule out the involvement of neutrophil/MPO-dependent pathway in nitrosative stress per se, because peroxynitrite formation as a result of the reaction of NO and O₂⁻ is a neutrophil-independent factor and may be able to mask the association between activated neutrophils and NT⁺ cells.

Finally, we showed a significant relationship between MPO⁺ cells and PaCO₂ level, and this may reflect an

upregulation of neutrophilic activity in airway-alveolar wall of severe COPD with the subsequent alveolar destruction followed by air trapping. Furthermore, an involvement of nitrosative stress on respiratory muscle dysfunction in patients with severe COPD may occur with consequent alveolar hypoventilation.³²

In vitro, the enzyme XO generates O₂⁻, H₂O₂, and peroxynitrite.³³ XO activity is elevated in sputum and BAL from patients with stable COPD compared with nonsmokers.^{34,35} Allopurinol (an XO inhibitor) decreases XO activity and NT formation.³⁵ In the current study focused on bronchial mucosa, we did not find any changes in XO⁺ cells among our study groups, indicating a lack of actions for XO in the nitrosative stress cascade in our biopsies. We may speculate that in this compartment, other oxidant-generating enzymes could be involved in the biochemical process forming reactive nitrogen species in patients with COPD, or alternatively, that changes in activity of XO could not be reflected by changes in its tissue expression. Barreiro et al³⁶ recently reported decreased eNOS and similar nitrotyrosine levels in the diaphragmatic muscle of patients with severe COPD compared with control subjects. These data are in contrast with our findings. However, the wide anatomic differences between the bronchial mucosa analyzed in our current study together with the lack of a significant inflammatory response (absence of detectable iNOS) in the diaphragmatic muscle may account for this discrepancy. These findings together indicate that the development of oxidative and nitrosative stress may be different in various tissues.

In conclusion, the bronchial mucosa in severe COPD is characterized by an advanced inflammatory process with the presence of neutrophils positive to MPO and by nitrosative stress with overexpressed NO synthase isoforms and NT formation probably involved in the impairment of airway function. Our study may have clinical relevance in the attempt to identify new pharmacological targets to reduce the nitrosative stress potentially involved in the pathogenesis of COPD.

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