Nitric Oxide Synthase Isoenzyme Expression and Activity in Peripheral Lung Tissue of Patients with Chronic Obstructive Pulmonary Disease

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ABSTRACT

Rationale: Nitric oxide (NO) is increased in the lung periphery of patients with chronic obstructive pulmonary disease (COPD). However, expression of the NO synthase(s) responsible for elevated NO has not been identified in the peripheral lung tissue of patients with COPD of varying severity.

Objectives:

Methods: Protein and mRNA expression of nitric oxide synthase type I (neuronal NOS [nNOS]), type II (inducible NOS [iNOS]), and type III (endothelial NOS [eNOS]) were quantified by Western blotting and reverse transcription-polymerase chain reaction, respectively, in specimens of surgically resected lung tissue from nonsmoker control subjects, patients with COPD of varying severity, and smokers without COPD, and in a lung epithelial cell line (A549). The effects of nitrative/oxidative stress on NOS expression and activity were also evaluated in vitro in A549 cells. nNOS nitration was quantified by immunoprecipitation and dimerization of nNOS was detected by low-temperature SDS–PAGE/Western blot in the presence of the peroxynitrite generator, 3-morpholinosydnonimine-N-ethy carbamide (SIN1), in vitro and in vivo.

Measurements and Main Results: Lung tissue from patients with severe and very severe COPD had graded increases in nNOS (mRNA and protein) compared with nonsmokers and normal smokers. Hydrogen peroxide (H₂O₂) and SIN1 as well as the cytokine mixture (IFN-γ, IL-1β, and tumor necrosis factor-α) increased mRNA expression and activity of nNOS in A549 cells in a concentration-dependent manner compared with nontreated cells. Tyrosine nitration resulted in an increase in nNOS activity in vitro, but did not affect its dimerization.

Conclusions: Patients with COPD have a significant increase in nNOS expression and activity that reflects the severity of the disease and may be secondary to oxidative stress.

KEYWORDS: nitric oxide synthase, nitrosative stress, nitration, chronic obstructive pulmonary disease
AT A GLANCE COMMENTARY
Scientific Knowledge on the Subject

Nitric oxide is increased in the peripheral lungs of patients with chronic obstructive pulmonary disease (COPD); however, the source is unclear.

What This Study Adds to the Field

Neuronal nitric oxide synthase expression and activity are increased in patients with COPD according to the severity of their disease, and this may be the cause of high levels of nitrate stress in peripheral lung.

Chronic obstructive pulmonary disease (COPD) is a major and increasing global health problem and there are no current therapies that reduce the inevitable progression of the disease. COPD is characterized by airflow limitation that is not fully reversible and is usually both progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases (1). Cigarette smoking is strongly linked to the ongoing inflammation in the airways and lung parenchyma, and the severity of airflow limitation is correlated with the degree of pulmonary inflammation (2, 3).

Reactive nitrogen species, which are contained in cigarette smoke and are also produced endogenously, have been implicated in the pathogenesis of COPD (4). Nitric oxide (NO) is a free radical able to interact with superoxide anions (O_2^−) to form the strong nitrosant peroxynitrite (ONOO−), which can lead to nitration (addition of −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 (5).

In asthma increased levels of exhaled NO are likely to be due to activation of the inducible form of NO synthase (iNOS or type II NOS) and increased iNOS expression and increased nitrotyrosine immunoreactivity have been reported (6). Treatment with corticosteroids in asthma results in a reduction of exhaled NO levels due to both reducing effects of steroids on the underlying airway inflammation and inhibitory effects on iNOS expression itself (6, 7).

Exhaled NO levels in COPD are not elevated as in asthma and may be reduced by cigarette smoking (8, 9). Furthermore, exhaled NO levels are not reduced by steroid treatment (10). Partitioning of exhaled NO in COPD by measurement at various expiratory flows has suggested that NO may originate from the peripheral lung compartment rather than the central airways (11). Furthermore, nebulized aminoguanidine, a weakly selective iNOS inhibitor, markedly reduces (∼90%) NO produced in the central airways (J_NO) in asthma (12), whereas in COPD the increased exhaled NO from peripheral lung (C_\text{alv}) is decreased by less than 50% (13). The increase in C_\text{alv} and exhaled ONOO− levels was not completely suppressed by nebulized aminoguanidine in patients with COPD (13), suggesting that an isoform(s) different from iNOS may also be involved in peripheral NO production in patients with COPD.

NO derived from various isoforms of NOS plays a critical and diverse physiological role and has been implicated in several diseases, including asthma and COPD (9). NO produced by neuronal NOS (nNOS, type I NOS) functions as a neurotransmitter of bronchodilator nerves, NO produced by endothelial NOS (eNOS, type III NOS) is a bronchial vasodilator and NO produced by iNOS is an effector in cytotoxicity and cytostasis and is involved in host defense (14–17).

The paradigm of constitutive and inducible NOS isoforms has been modified from its original concept (18). In particular, although nNOS and eNOS are constitutively expressed—and indeed are
sometimes collectively referred to as constitutive NOS (cNOS)—it is now apparent that their activity can be regulated by various factors. Conversely, iNOS may be constitutively expressed at certain sites. It is therefore important to determine the levels of NOS isoforms expressed in various organs and tissues to estimate the amount of NO produced and also to understand the in vivo actions of the individual NOS isoforms.

The source of exhaled NO is unclear, although increased expression of iNOS has been reported with elevated exhaled NO in asthmatic airways and has been localized to the airway epithelium and infiltrating inflammatory cells (19). Much of the work regarding the regulation of iNOS expression has been performed in murine models. In these systems iNOS is readily induced and produces large quantities of NO. However, induction of human iNOS has proved to be more complex (20).

An increase in iNOS expression has been reported in the bronchi of patients with COPD (21), but there are no studies on the expression and regulation of NOS isoforms in the peripheral lung tissue of patients with COPD.

In this study we investigated the protein and mRNA expression of the three NOS isoenzymes in the peripheral lung tissue of patients with COPD and also studied the impact of nitrative/oxidative stress on NOS expression and activity in vitro in A549 cells, which are derived from alveolar epithelial cells.

METHODS

Subjects

The guidelines for grading disease severity in COPD according to the Global Initiative for Chronic Obstructive Lung Disease (1) were followed. Specimens of lung tissue and data on patient lung function were obtained from a tissue bank that was linked to an established patient registry (22). Specimens of peripheral lung tissue were obtained from 11 patients who were nonsmoker control (NSC) subjects without symptoms and had normal lung function, and from 6 smoker control (SC) subjects and 17 patients who were smokers: 4 with stage 1 COPD, and 11 with stage 2 COPD. Specimens of peripheral lung tissue were obtained from an additional 7 patients with stage 3–4 COPD who were undergoing lung volume reduction surgery. All subjects provided written informed consent for the deposition of their tissues in the tissue bank from which we obtained the specimens and for their use in research studies of this type.

Cell Culture

Human lung epithelial (A549) cells were grown in culture medium (Dulbecco's modified Eagle's medium) to approximately 80–90% confluence and then cultured for 24 hours in additive-free medium before experimental treatments. A549 cells were treated with cytokine mixture (CytoMix; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) (IL-1β, 10 ng/ml; tumor necrosis factor-α, 10 ng/ml; IFN-γ, 100 ng/ml) or LPS (1 μg/ml), with a peroxynitrite generator (3-morpholinosydnonimine-N-ethylcarbamide [SIN1] at 10, 100, and 500 μM) and with hydrogen peroxide (H₂O₂ at 1, 10, and 100 μM) and incubated for 8 and 24 hours to determine mRNA expression.

Additional details on the method are provided in the online supplement.

Preparation of Cell Extracts
Peripheral lung tissue specimens (three pieces approximately 0.5 cm$^3$ in size) were ground under liquid nitrogen, using a pestle and mortar. Hypotonic buffer (10 mM N-2-hydroxyethylpiperazine-N’-ethane sulfonic acid–sodium hydroxide [pH 7.9], 1.5 mM magnesium chloride, 10 mM potassium chloride, 10 mM 2-mercaptoethanol, and one protease inhibitor cocktail tablet per 10 ml [Roche Diagnostics, Lewes, UK]) was added to samples to remove red blood cells and secretions and to loosen cell membranes, and then left for 15 minutes on ice. A549 cells were washed with ice-cold Hanks' balance salt solution twice and scraped off. The cells were collected by spinning down at 12,000 rpm for 3 minutes at 4°C.

The whole cell extraction from peripheral lung tissue or A549 cells was performed on ice. The radioimmunoprecipitation assay (RIPA) buffer, which contains 50 mM TRIS-HCl (pH 7.4), 2% Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, 150 mM NaCl, deionized water, and a protein inhibitor tablet (Roche Diagnostics, Mannheim, Germany), was prepared as previously reported (23). Samples were resuspended in RIPA buffer and left for 20 minutes on ice, and then sonicated with a Vibra-Cell high-density ultrasonic processor (Jencons, Leicestershire, UK) for 10 seconds at an amplitude of 60. Samples were then centrifuged again at 12,000 rpm for 10 minutes at 4°C. The supernatant was immediately transferred into fresh cold labeled microcentrifuge tubes and the protein concentration of the cell lysate was determined with a Bradford protein assay kit, using bovine serum albumin as a standard (Bio-Rad, Hemel Hempstead, UK).

Detection of NOS Protein Expression

Protein samples were analyzed by SDS–PAGE and Western blot analysis with the use of an immunoblot apparatus (XCell SureLock mini-cell and blot module kit; Invitrogen, Carlsbad, CA). Immunoreactive bands were detected by an enhanced chemiluminescence technique (ECL solution; Amersham Ltd, Amersham, UK) using specific antibodies. Variation of band density of NOS isoforms in different samples was corrected for the band density of β-actin (Abcam, Cambridge, UK).

Additional details on the method are provided in the online supplement.

mRNA Expression: Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from approximately 1 × 10$^6$ A549 cells or two specimens of lung tissue of 0.3 cm$^3$, using an RNeasy kit (Qiagen, Crawley, UK) and reverse transcription to obtain cDNA was performed with the use of an Omniscript RT kit (Qiagen) as previously described (24). Gene transcript levels of nNOS, iNOS, and eNOS were quantified by real-time polymerase chain reaction (PCR) using a TaqMan system (Applied Biosystems, Foster City, CA) on a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia). Variation in transcript amounts in different samples was corrected with glyceraldehydes-3-phosphate dehydrogenase expression. Sequences of the primer pairs used for the PCR are given in the online supplement.

Immunohistochemistry

The tissue samples had been fixed in 10% buffered formalin and embedded in paraffin, and cut. The immunostaining was performed with an EnVision G2 system/AP, rabbit/mouse (DakoCytomation, Cambridge, UK). Comparison of immunoreactivities was conducted between three patients with COPD and three NSC subjects and immunostaining was evaluated by two blinded investigators. Negative control slides were exposed only to secondary antibodies.

Additional details on the method are provided in the online supplement.
Cell Viability

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. None of the treatments used in this study altered cell viability when compared with untreated cells.

Additional details on the method are provided in the online supplement.

nNOS Activity Assay

Whole cell extract of A549 cells was prepared by homogenization and incubated with an nNOS-specific substrate (N-cyclopropyl-N^1-hydroxyguanidine-HCl, 5 nM; Alexis Biochemicals, AXXORA Ltd, Nottingham, UK) for 1 hour at 30°C; released nitrite was measured to determine nNOS activity.

Nitrite/Nitrate Assay

Total nitrite released was measured with a nitrate/nitrite colorimetric assay kit (lactate dehydrogenase method) (AXXORA Ltd).

Nitration of nNOS

To test whether nNOS was a nitrated protein, two experiments were performed: in vitro and in vivo.

An nNOS human recombinant (10 μl) (AXXORA) was incubated with SIN1 (10 and 100 μM) in presence of NaHCO₃ (200 mM) for 1 hour at 30°C and nitrated nNOS was detected with mouse monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Charlottesville, VA). Release of nitrite was quantified to evaluate nNOS activity.

nNOS was immunoprecipitated by the addition of 5 μg of anti-nNOS rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in whole cell extracts of A549 cells. The immune complex was incubated for 4 hours at 4°C. Protein A/G PLUS agarose beads (30 μl) (50% slurry; Santa Cruz Biotechnology) was added to the mixture to collect the nNOS immune complex or nitrated protein immune complex. The immune complex was then washed with RIPA solution (50 mM TRIS-HCl [pH 7.4], 2% [vol/vol] Nonidet P-40, 0.25% [wt/vol] sodium deoxycholate, 150 mM NaCl, and deionized water) three times and incubated with 30 μl of RIPA. Peroxynitrite was added at various concentrations (10, 100, and 500 μM). Western blotting was performed and bands were detected with anti-nNOS and anti-nitrotyrosine antibodies.

Detection of Dimerization of NOS by Low-Temperature SDS–PAGE/Western Blot

Recombinant nNOS was incubated with SIN1 (10 and 100 μM) and NaHCO₃ (200 mM) or with distilled water and left for 30 minutes at 34°C. Laemmli buffer was added and each sample was divided into two, one of which was boiled. Low-temperature SDS—PAGE/Western blot was performed according to the procedures of Klatt and colleagues (25). Gels and buffers were equilibrated at 4°C before electrophoresis and the buffer tank was placed in an ice bath during the electrophoresis in a cold room to maintain the temperature of the gel below 15°C. The membrane was probed with anti-nNOS antibody and reprobed with anti-nitrotyrosine antibody.

Statistical Analysis
Data are presented as means ± the standard deviation (SD) or standard error of the mean (SEM). Analysis of variance was performed with the use of the nonparametric Kruskal–Wallis test. When the result was significant, the Mann-Whitney U test was performed for comparisons between groups (SPSS software; SPSS, Chicago, IL). Correlation coefficients were calculated with the use of Spearman’s rank method. A P value of less than 0.05 was considered to indicate statistical significance. All reported P values are two-sided.

RESULTS

Baseline characteristics of the patients are summarized in Table 1.

TABLE 1

SUBJECT CHARACTERISTICS.

NOS mRNA and Protein Expression in Lung Tissues

There was a significant increase in iNOS mRNA expression (P < 0.05) in samples from patients with stage 2 disease (65.2 ± 23) and the values were also higher in peripheral lung tissue from patients with more severe COPD (stage 3, 79.1 ± 13.2; stage 4, 78.9 ± 22.3) compared with samples from NSC subjects (12.5 ± 6.6). There were no significant differences in the levels of iNOS mRNA between samples from SC subjects (22.2 ± 8.9), patients with stage 1 disease (17.2 ± 12.6), or from NSC subjects (Figure 1A).

Figure 1

Figure 1. Messenger RNA expression of nitric oxide synthase (NOS) isoenzymes in human peripheral lung tissue from nonsmoker control (NSC) subjects, smoker control (SC) subjects, and patients with chronic obstructive pulmonary disease (COPD) at Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage 1, 2, 3, or 4. The results are plotted as means ± SEM; *P < 0.05 versus nonsmokers. (A) iNOS mRNA expression; (B) nNOS mRNA expression; (C) eNOS mRNA expression. eNOS = endothelial nitric oxide synthase; iNOS = inducible nitric oxide synthase; nNOS = neuronal nitric oxide synthase.

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iNOS protein expression levels reached significance (P < 0.05) in samples from patients with stage 1 disease (1.2 ± 0.1, iNOS/β-actin) and patients with stage 2 disease (1.8 ± 0.2) as compared with those from NSC subjects (0.6 ± 0.2); iNOS protein expression levels did not show a significant difference at stage 3 (1.9 ± 0.5) and stage 4 (1.8 ± 0.3) disease compared with levels from patients with stage 1 and 2 disease, although the values were increased (P < 0.05) compared with those of NSC and SC subjects (0.9 ± 0.2) (Figure 2A).

Figure 2. Protein expression of nitric oxide synthase (NOS) isoenzymes in peripheral lung tissue from nonsmoker control (NSC) subjects, smoker control (SC) subjects, and patients with chronic obstructive pulmonary disease (COPD) at Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage 1, 2, 3, or 4. The samples were analyzed in a blinded manner and the samples from each subject group of each disease stage were randomly distributed on the blots. Therefore the representative image of each disease stage has been selected and provided. The statistical analysis of density was determined from the original gels and the data were not corrected. Densitometric analyses are normalized to β-actin and expressed as means ± SEM. *P < 0.05 versus nonsmokers; **P < 0.0001 versus nonsmokers. (A) iNOS protein expression; (B) nNOS protein expression; (C) eNOS protein expression; (D) correlation between nNOS protein expression levels and FEV₁ (% predicted) in all study groups; (E) correlation between nNOS protein expression levels and FEV₁/FVC (% predicted) in all study groups; (F) correlation between nNOS protein expression levels and FEV₁ (% predicted) only in patients with COPD; (G) correlation between nNOS protein expression levels and FEV₁/FVC (% predicted) only in patients with COPD. eNOS = endothelial nitric oxide synthase; iNOS = inducible nitric oxide synthase; nNOS = neuronal nitric oxide synthase.

Protein expression of nitric oxide synthase (NOS) isoenzymes in peripheral lung tissue from nonsmoker control (NSC) subjects, smoker control (SC) subjects, and patients with chronic obstructive pulmonary disease (COPD) at Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage 1, 2, 3, or 4.

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Increased nNOS mRNA expression reached significance (P < 0.05) in peripheral lung tissue from patients with stage 1 disease (416.4 ± 76.2) and stage 2 disease (477.1 ± 43.3) as compared with those from NSC subjects (211.6 ± 54) and from SC subjects (261.3 ± 45.2); nNOS mRNA levels were also higher from patients with stage 3 disease (677.2 ± 19) and stage 4 disease (642.3 ± 29), although the levels were not significantly different between the two groups (Figure 1B).

A similar trend with nNOS mRNA was also noted in the protein expression levels for nNOS: there were no significant differences in the levels of nNOS between samples from SC subjects (0.85 ± 0.2), patients with stage 1 disease (0.75 ± 0.24), and samples from NSC subjects (0.78 ± 0.21); the differences became significant between samples from NSC subjects and those from patients with stage 2 disease (1.3 ± 0.18; P < 0.05), stage 3 disease (4.2 ± 1.6; P < 0.05), and stage 4 disease (3.9 ± 0.7; P < 0.0001) (Figure 2B). Lung samples from SC subjects had lower nNOS levels compared with those from patients with stage 3 disease (P < 0.0001) and stage 4 disease (P < 0.0001) and samples from patients with stage 2 disease had lower levels (P < 0.0001) compared with samples from patients with stage 3 and 4 disease.

No differences were found in eNOS mRNA levels between NSC subjects (101 ± 48) and SC subjects (148.8 ± 76.6) and patients with COPD at stage 1 (157.3 ± 48.6) and stage 2 (135.5 ± 35), but a significant reduction was found at stage 3–4 (17.6 ± 9.5; P < 0.05) (Figure 1C).

A similar trend with eNOS mRNA was noted in the eNOS protein expression levels: there were no significant differences in the levels of eNOS between samples from SC subjects (0.5 ± 0.07), patients with stage 1 disease (0.5 ± 0.04), stage 2 disease (0.4 ± 0.04), stage 3 disease (0.37 ± 0.09), and samples from NSC subjects (0.4 ± 0.07); the differences became significant with samples from patients with stage 4 disease (0.13 ± 0.02; P < 0.05) (Figure 2C).

nNOS protein expression was correlated with FEV1 (% predicted) (r = −0.7, P < 0.0001) and FEV1/FVC (r = −0.65, P < 0.0001) in a Spearman correlation analysis (Figures 2D and 2E, respectively) in all studied groups. Interestingly, these correlations were preserved when healthy subjects were excluded from the analysis (Figures 2F and 2G, respectively). No correlations were found between iNOS or eNOS and lung function parameters.

Immunohistochemistry showed that the alveolar epithelium in peripheral lung tissue from patients with COPD was strongly positive for nNOS (Figure 3C), whereas specimens of healthy control subjects exhibited only a little immunoreactivity (Figure 3B).
Figure 3. Immunohistochemical localization of neuronal nitric oxide (NO) synthase expression in peripheral lung images of lung tissue. Representative photomicrographs show the alveolar epithelium from (A) a negative control subject (original magnification, ×100), (B) a healthy control subject (original magnification, ×200), and (C) a patient with chronic obstructive pulmonary disease (COPD) (original magnification, ×200) immunostained for identification of neuronal nitric oxide synthase (red color). Inset: Alveolar epithelium at a higher magnification. Arrows indicate representative positively stained cells.

Immunohistochemical localization of neuronal nitric oxide (NO) synthase expression in peripheral lung images of lung tissue. [More]

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Effect of Reactive Oxygen Species/Reactive Nitrogen Species on nNOS Expression and Activity in A549 Cells

The effect of H\textsubscript{2}O\textsubscript{2} and SIN1 on nNOS expression was evaluated in A549 cells in vitro. H\textsubscript{2}O\textsubscript{2} and SIN1 concentration-dependently increased nNOS mRNA expression (H\textsubscript{2}O\textsubscript{2}: 1 μM, 0.018 ± 0.003; 10 μM, 0.030 ± 0.0015; 100 μM, 0.07 ± 0.01, SIN1: 10 μM, 0.021 ± 0.002; 100 μM, 0.028 ± 0.005; 500 μM, 0.051 ± 0.006) and similar results were seen with cytomix (0.067 ± 0.01), compared with nontreated cells (0.003 ± 0.004) (Figure 4A).

Figure 4. Effect of reactive oxygen and nitrogen species on nitric oxide synthase (NOS) isoform expression and activity in A549 cells. A549 cells were cultured for 8 hours in the absence or presence of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}, at 1, 10, and 100 μM), 3-morpholinosydnonimine-N-ethylcarbamide (SIN1, at 10, 100, and 500 μM), and cytomix (Cyt: IFN-γ [100 μg/ml], tumor necrosis factor-α [10 μg/ml], IL-1β [10 μg/ml], and LPS [1 μg/ml]). (A) Neuronal nitric oxide synthase (nNOS) mRNA expression normalized to glyceraldehydes-3-phosphate dehydrogenase (GAPDH); values are plotted as means ± SD. *P < 0.05; **P < 0.001 versus nonstimulated cells. Data are representative of n = 3 experiments. (B) nNOS protein expression levels normalized to β-actin; values are plotted as means ± SD. *P < 0.05; **P < 0.001 versus nonstimulated cells. Data are representative of n = 3 experiments. The samples were analyzed in a blinded manner and the samples from each treatment were randomly distributed on the blots. Therefore the representative image of each treatment has been selected and provided. (C) nNOS activity measured with an
nNOS-specific substrate, with measurement of nitrite formation. Values are shown as means ± SD. *P < 0.05; **P < 0.001 versus nonstimulated cells. Data are representative of n = 3 experiments.

Effect of reactive oxygen and nitrogen species on nitric oxide synthase (NOS) isoform expression and activity in A549 cells. [More]

Effect of reactive oxygen and nitrogen species on nitric oxide synthase (NOS) isoform expression and activity in A549 cells. A549 cells were cultured for 8 hours in the absence or presence of hydrogen peroxide (H2O2, at 1, 10, and 100 μM), 3-morpholinosydnonimine-N-ethylcarbamide (SIN1, at 10, 100, and 500 μM), and cytomix (Cyt: IFN-γ [100 μg/ml], tumor necrosis factor-α [10 μg/ml], IL-1β [10 μg/ml], and LPS [1 μg/ml]). (A) Neuronal nitric oxide synthase (nNOS) mRNA expression normalized to glyceraldehydes-3-phosphate dehydrogenase (GAPDH); values are plotted as means ± SD. *P < 0.05; **P < 0.001 versus nonstimulated cells. Data are representative of n = 3 experiments. (B) nNOS protein expression levels normalized to β-actin; values are plotted as means ± SD. *P < 0.05; **P < 0.001 versus nonstimulated cells. Data are representative of n = 3 experiments. The samples were analyzed in a blinded manner and the samples from each treatment were randomly distributed on the blots. Therefore the representative image of each treatment has been selected and provided. (C) nNOS activity measured with an nNOS-specific substrate, with measurement of nitrite formation. Values are shown as means ± SD. *P < 0.05; **P < 0.001 versus nonstimulated cells. Data are representative of n = 3 experiments. [Minimize]

As shown in Figure 4B, protein expression of nNOS was also increased by stimulation with H2O2 (0.28 ± 0.02), SIN1 (0.44 ± 0.03), and cytomix (0.22 ± 0.04) when normalized to β-actin protein as endogenous standard.

nNOS activity in cell extracts, which was measured with the nNOS-selective substrate N-cyclopropyl-N1-hydroxyguanidine-HCl, increased after stimulation with SIN1 (10 μM, 0.033 ± 0.0028; 100 μM, 0.0395 ± 0.005) for 24 hours compared with nonstimulated cells (0.016 ± 0.003). H2O2 also induced nNOS activity in a concentration-dependent manner (10 μM, 0.029 ± 0.006; 100 μM, 0.036 ± 0.006). Cytomix also increased nNOS activity (0.027 ± 0.003) (Figure 4C). However, SIN1 increased nNOS activity to a greater extent than mRNA expression when compared with the effects of cytomix.

Nitration of nNOS

As shown in Figure 5A, 3-nitrotyrosine was increased in recombinant nNOS after SIN1 treatment, suggesting that nNOS is a nitrated protein (SIN1: 10 μM, 15% increased; 100 μM, 22.3% increased). Immunoprecipitated nNOS in cells treated with peroxynitrite was clearly nitrated by peroxynitrite in a concentration-dependent manner (10 nM, 10.5% increase in nitrotyrosine positivity; 100 nM, 36.8% increase; 500 nM, 73.7% increase) (Figure 5B). This increase in nNOS nitration paralleled an increase in nNOS activity in the in vitro experiment with recombinant nNOS (Figure 5C) and in A549 cells (NS: 0.03 ± 0.01; SIN1, 100 μM: 0.096 ± 0.026) (Figure 5D).
Figure 5. Nitration and dimerization of neuronal nitric oxide synthase (nNOS). (A) Human recombinant nNOS was incubated without or with 3-morpholinosydnonimine-N-ethylcarbamide (SIN1; 10 and 100 μM) in the presence of NaHCO3 (200 mM) for 1 hour at 30°C and nitrated nNOS was detected with an anti-nitrotyrosine antibody. The blot is representative of n = 3 experiments. (B) nNOS was immunoprecipitated from A549 cells by the addition anti-nNOS antibody and the immunocomplex was incubated without or with peroxynitrite (PN) at various concentrations (nanomolar) and bands were detected with nitrotyrosine and an nNOS antibody. Columns graphically represent the densitometric values and are representative of n = 3 experiments. Data are expressed as means ± SEM. (C) Human recombinant nNOS was incubated with or without SIN1 in the presence of NaHCO3 (200 mM) for 1 hour at 30°C and nNOS activity was measured with the substrate N-cyclopropyl-N1-hydroxyguanidine-HCl. Data are representative of three experiments and the columns and error bars represent means ± SEM. (D) nNOS was immunoprecipitated from A549 cells by the addition of anti-nNOS rabbit polyclonal antibody and the resulting immunocomplex was subjected to nitration by incubation with NaHCO3 (200 mM) and SIN1 (500 μM) or exposed to NaHCO3 (200 mM) alone as a control. nNOS substrate was added and the release of nitrite was determined to quantify nNOS activity. Columns and error bars represent means ± SEM and are representative of three experiments. (E) Endothelial nitric oxide synthase (eNOS) expression in denatured/nondenatured samples in the presence and absence of SIN1. Bands corresponding to higher molecular weight represent eNOS dimers and the bands of lower molecular weight to eNOS monomers. (F) nNOS expression in denatured/nondenatured samples in the presence and absence of SIN1. Bands corresponding to higher molecular weight represent nNOS dimers and the bands of lower molecular weight to nNOS monomers.

Nitration and dimerization of neuronal nitric oxide synthase (nNOS) [More]

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Dimerization of NOS

Dimerized eNOS was found under nondenaturing conditions and this dimerization was abolished under denaturing conditions (Figure 5E). The dimer-to-monomer ratio as determined by densitometry was 4.6 at baseline and was reduced by SIN1 treatment to 1.4, indicating that SIN1 reduces dimerization of eNOS. By contrast, although nNOS was also dimerized at baseline (Figure 5D).
5F), SIN1 did not affect nNOS dimerization (dimer-to-monomer ratio, 0.98 and 1.07 in the absence and in the presence of SIN1, respectively).

**DISCUSSION**

We have demonstrated the expression of all three isoforms of NOS in human peripheral lung, both at the mRNA and protein levels. In the peripheral lung tissue of patients with COPD, iNOS mRNA expression was increased at earlier stages of the disease (GOLD stages 1 and 2) but decreased in the patients with the most severe disease (GOLD stages 3 and 4). Protein expression also increased at earlier stages of COPD, in agreement with mRNA expression, but was not reduced at later stages of the disease.

Also, proinflammatory cytokine-induced iNOS expression was decreased by oxidative stress, such as hydrogen peroxide (H$_2$O$_2$), cigarette smoke–conditioned medium (CSM), and the peroxynitrite generator SIN1 (data not shown), consistent with findings in severe COPD, in which there is a high level of oxidative stress and peroxynitrite formation (13, 26, 27).

It is important to note that some of the patients were treated with inhaled corticosteroids, but there did not appear to be any obvious difference in iNOS between patients who were treated with corticosteroids. In any case, it is unlikely that steroids would inhibit iNOS in patients with COPD, as there is no effect of high-dose inhaled steroid on exhaled NO in patients with COPD (11). Furthermore, it is likely that the inflammation that induces iNOS is steroid resistant in patients with COPD.

Expression of eNOS mRNA and protein, which occurs mainly in epithelial and endothelial cells, did not change in mild to moderate COPD, but was reduced in the more severe stages of COPD. This might be a reflection of the destruction of alveoli, which leads to a loss of epithelial and endothelial cells in these patients as a result of emphysema. Despite the eNOS/iNOS reduction in severe COPD, we have previously shown an increase in exhaled NO derived from peripheral lung (Calv) in COPD in GOLD stages 2 and 4 (11). In the present study, nNOS protein and mRNA expression increased with severity of disease and there was a significant increase at stages 2, 3, and 4, with a positive correlation between nNOS protein expression and disease severity as measured by the percentage of predicted FEV$_1$ and with the degree of airway obstruction as measured by the FEV$_1$/FVC ratio. Our results suggest that the main source of Calv is likely to be nNOS in the severe stages of the disease, whereas iNOS is involved in NO elevation mainly in the less severe stages of COPD. Barreiro and colleagues have also reported an elevation of nNOS in skeletal muscle of patients with COPD, suggesting that there may be common mechanisms for its elevation in peripheral lung and skeletal muscle (28). In the present study, the alveolar epithelium showed nNOS immunoreactivity in COPD. Furthermore, an alveolar type II cell line, A549, clearly expressed nNOS as well as iNOS isoforms.

The present study showed that nNOS expression was induced by nitrative stress and oxidative stress as well as inflammatory cytokine stimulation. Jang and colleagues have previously demonstrated nNOS expression in the lung after ozone exposure, but not after allergen exposure, and it is interesting that this was accompanied by a reduction in iNOS expression, consistent with our findings in patients with COPD (29). Higher levels of oxidative and nitrative stress are seen in patients with COPD (21) and we now report an increase in nNOS expression. Li and colleagues (30) showed that acetylated NF-κB preferably bound to the promoter of nNOS although nonmodified NF-κB is a well-known iNOS inducer. Nitrative stress and oxidative stress induce a defect of SIRT1 (sirtuin 1), which deacetylates acetylated NF-κB (31). Thus, oxidative stress/nitrative stress induces acetylated NF-κB due to SIRT1 reduction.
Increased oxidative/nitrative stress seen in the periphery of the lung of patients with COPD may account for the increased expression of nNOS in alveolar epithelial cells, thus contributing to the increase in Calv. However, more importantly, we found that nNOS activity is higher in nNOS mRNA expression under nitrative stress. We found that SIN1 increased nNOS activity to a greater extent than inflammatory cytokines despite a more potent induction of nNOS mRNA by cytomix than SIN1 at 100 nM. This suggests that the increase in nNOS activity is due at least in part to some posttranscriptional modification and that the increase in nNOS may involve not only increased gene expression but also increased enzyme activity.

The activity of nNOS is dependent on several cofactors, such as calmodulin and flavins. The binding of the calcium–calmodulin complex in the presence of oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) activates the enzyme to convert l-arginine to NO. Recognition sites for NADPH, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) have been identified within the nNOS molecule (32). Biochemical studies have demonstrated that FAD and FMN bind in stoichiometric amounts to nNOS. The reaction mechanism is believed to involve reduction of FAD by NADPH, which then reduces FMN, which in turn transfers electrons to the ferric heme moiety, allowing an interaction with molecular oxygen. It has been shown that dimerization is required for the catalytic activity of all three NOS isoforms (33, 34). The process of dimerization appears to be dependent on the presence of the cofactors heme and tetrahydrobiopterin (BH4), as well as the enzyme substrate l-arginine. These factors are thought to associate with the N-terminal, heme-binding region of the protein. However, it is unclear whether sequences within the reductase domain are also important for dimerization. Ravi and colleagues (35) showed that in the eNOS enzyme intrasubunit cysteine and the tetrathiolate cysteine residues are important for dimer maintenance and enzyme activity; the loss of zinc due to tetrathiolate cluster oxidation by NO at the dimeric interface may lead to altered pterin binding, making the enzyme unstable. This effect in combination with intrasubunit nitrosylation may lead to dimer collapse. In our study nNOS was found to be nitrated and nitration of nNOS preserves its activity, in contrast to eNOS activity, which has been shown to be abolished by nitration (35). In fact, nitration of eNOS inhibited its dimerization whereas nitration of nNOS had no effect on dimerization. iNOS activity was also found to be reduced by oxidative stress. Thus, as nNOS activity was not affected by nitratative stress, in contrast to eNOS and iNOS, this suggests that nNOS is predominantly involved in the NO production seen in peripheral lung tissue of patients with severe COPD.

In addition, we speculate that nitration itself might increase its activity. Although many of the proteins such as IL-8, histone deacetylase, and manganese-superoxide dismutase were reported to reduce their activity after nitration (36, 37), matrix metalloproteinase-3 activity is reported to be enhanced by nitration (38). The tyrosine residues nitrated in nNOS have not yet been identified, however. We have not yet explored the molecular mechanisms and signal transduction pathways whereby oxidative and nitrative stress may affect nNOS activity; this is beyond the scope of this study but an area for future research.

An increase in lung nNOS expression in patients with severe COPD in combination with increased superoxide anion formation would result in the local formation of peroxynitrite and nitration of tyrosine residues on several target proteins.

Although it is difficult to assess the functional relevance of our findings, two hypotheses are proposed:

An increase in nNOS expression may be a compensatory mechanism, as in some experimental systems nNOS has been found to have a protective and antiinflammatory effect.

An increase in lung nNOS expression in patients with severe COPD is a pathological process whose combination with excessive superoxide anion leads to the formation of the highly


reactive oxidant peroxynitrite and the development of nitrosative stress.  

This speculation is supported by the elevated NO levels in peripheral lungs, elevated peroxynitrite levels in exhaled breath condensate, and elevated nitrotyrosine levels in patients with COPD (13, 21, 39). This, along with the absence of abundant iNOS expression in more severe COPD, suggests that an increase in nNOS expression may contribute significantly to NO and peroxynitrite production in peripheral lungs. 

De Sanctis and colleagues showed that nNOS contributes significantly to the NO in exhaled air and that NO derived from nNOS contributes to baseline airway responsiveness and increased airway responsiveness after allergen challenge in mice (40). Jang and colleagues demonstrated that eNOS and nNOS isoforms induce airway responsiveness in mice after ozone exposure, whereas iNOS is decreased, suggesting that eNOS and nNOS contribute to the formation of NO metabolites in mice after ozone exposure (29, 41). Grasemann and colleagues investigated the existence of a genetic association between a polymorphism in the nNOS gene and the diagnosis of asthma, concluding that nNOS may be a candidate gene for asthma (42). In human lung nNOS is expressed predominantly in capillary endothelial cells of alveolar septa and may play a role as an endothelium-derived regulator of capillary permeability, a modulator of cholinergic neuronal transmission, or an inhibitor of platelet aggregation (43).

Our data support the hypothesis that cNOS is involved in the regulation of nitrosative stress and on the basis of this hypothesis, although the high levels of iNOS-derived NO should be considered as a marker of inflammatory activity in the disease, an opposing effect could be played by the levels of cNOS-derived NO (i.e., having a protective role). The existence of a microenvironment-dependent and biochemical model of NO regulation is based on different levels of NOS expression and activation and different responses to NO. This model should be considered when predicting the progression and/or natural history of the disease in individual patients and when identifying new therapeutic strategies aimed at selectively potentiating the cNOS pathway and reducing the amplified inflammatory “iNOS arginase cascade.”

Unfortunately, we used archived lung from a tissue bank and were not able to measure C_{alv} in this study, and thus cannot correlate this with nNOS expression.

Further light may be shed on this question when highly selective iNOS and nNOS inhibitors become available for clinical studies.

We do believe that nNOS might contribute to COPD pathogenesis, although this is speculation that cannot be explored further until clinical trials with selective nNOS inhibitors are conducted. On the basis of data from animal studies using nNOS knockout mice (44), NO derived from nNOS likely contributes to airway disease pathogenesis. We should emphasize that our study does not exclude the possibility that an additional mechanism other than peroxynitrite might be involved in nNOS activation. The exact mechanisms responsible for the elevation of nNOS expression in the peripheral lung tissue of patients with severe COPD are unknown. A possible mechanism may involve pathological conditions in which proinflammatory cytokines and mediators are elevated, where an increase in nNOS expression may be expected.

Thus, increased production of NO in the peripheral lung tissue of patients with COPD depends mainly on nNOS, and may account for the increased production of peroxynitrite, thus amplifying nitrosative stress that further increases the expression of NOS isoforms responsible for the increased production of NO. This vicious cycle of nitrosative stress with a continuous balance/imbalance between nNOS and iNOS might be involved in the inflammation and progression of the pathogenesis of COPD.
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