Role of nitric oxide synthase/arginase balance in bronchial reactivity in patients with chronic obstructive pulmonary disease

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Tadié J-M, Henno P, Leroy I, Danel C, Naline E, Faisy C, Riquet M, Levy M, Israël-Biet D, Delclaux C. Role of nitric oxide synthase/arginase balance in bronchial reactivity in patients with chronic obstructive pulmonary disease. Am J Physiol Lung Cell Mol Physiol 294: L489–L497, 2008. First published August 3, 2007; doi:10.1152/ajplung.00109.2007.—Competition between nitric oxide synthases (NOSs) and arginases for their common substrate L-arginine is a highly conserved signaling messenger in a wide variety of physiological and pathological processes. NO and L-citrulline are produced by the nitric oxide synthases (NOSs) from L-arginine. Endothelial, epithelial, and smooth muscle cells can also metabolize L-arginine to urea and L-ornithine via arginases (9, 15, 39). Accordingly, the three NOS isoforms are expressed in conducting airways (mainly in epithelial cells), and all cellular subtypes of airway wall express at least one NOS isoform. Arginase expression in the airways has also been shown. Importantly, bronchial smooth muscle cells seem to express NOS and arginase isoforms. L-Ornithine is required for proline and polyamine synthesis, which are necessary for cellular proliferation. An increase in arginase activity is critical for the resolution of inflammatory injury and repair of tissue damage. However, polyamines and proline produced by L-ornithine metabolism may also be involved in bronchial and vascular remodeling in chronic inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD). By contrast, NO synthesis decreases smooth muscle proliferation; consequently, arginase and NOS may have opposing effects on tissue remodeling (17). The first aim of this study was to evaluate the expression of this enzymatic balance (NOSs and arginases) in bronchial tissue of patients without and with early COPD, before the occurrence of an obvious remodeling process.

The presence of airway hyperresponsiveness to methacholine is a predictive marker of airway remodeling (airflow limitation) in patients with COPD (24). The role of NOS/arginase balance in the control of airway tone is still debated. Besides the well-established effect of NO in mediating a protective effect on acetylcholine (ACh)-induced bronchoconstriction via its release by inhibitory nonadrenergic noncholinergic (NANC) nerves due to NOS1 activity (35), the bronchodilator role of NO released from other cellular origins and/or NOS remains the subject of debate, inasmuch as experiments using knockout mice have given conflicting results (30). Because of these Janus-faced properties, opposite therapeutic strategies are currently developed in bronchial diseases such as asthma or COPD. Compounds releasing NO together with specific inhibitors of NOS2 are currently under investigation (19, 29). Ricciardolo and colleagues (30) have suggested that during asthma the effect of high doses of corticosteroids is renewal of NOS3 activity by suppression of NOS2 expression. This latter hypothesis reconciles the apparently opposed therapeutic strategies aimed at restoring constitutive NO activity by specific inhibition of NOS2 activity. Along this line, Prado and colleagues (26) have recently demonstrated in a guinea pig model of asthma that specific blockade of NOS2 by 1400W...
attenuates bronchoconstriction and inflammatory and remodeling processes. It may be hypothesized that increased NOS2 expression could have a detrimental effect on airway reactivity. Consequently, the second aim of this study was to evaluate the ex vivo effects of NOS and arginase inhibition on bronchoconstrictive response to ACh.

**MATERIALS AND METHODS**

**Drugs.** Drugs used were ACh HCl (Pharmacie Centrale des Hôpitaux, Paris, France); N^G^-nitro-L-arginine methyl ester (L-NAME, nonspecific inhibitor of NOS) and L-arginine (Sigma, St. Louis, MO); 1400W (specific NOS2 inhibitor) and N^ω^-propyl-L-arginine (specific NOS1 inhibitor) (Cayman Chemical, Ann Arbor, MI); and (S)-(2-boronoethyl)-L-cysteine (BEC, inhibitor of arginases) and N^ω^-hydroxy-nor-L-arginine (nor-NOHA, inhibitor of arginases) (Calbiochem, EMD Chemical, San Diego, CA).

**Patients.** Patients referred for lung surgical procedures were eligible for this study. Depending on smoking history, pulmonary function test results, and histological analysis, the patients were classified as control (never a smoker or ex-smoker < 10 pack/yr; forced expiratory volume in 1 s (FEV1) > 80% and FEV1/forced vital capacity (FVC) > 75%; benign histology or lung cancer not associated with tobacco) or COPD subjects according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria. All patients quit smoking at least 3 mo before the surgical procedure and gave informed consent to these biological experiments according to the new French law (Bioethics Law of August 2004). This study was approved by our Institutional Review Board.

**Human bronchial tissue preparation.** Bronchial tissue was obtained at the time of the surgical procedure. Just after resection, segments of bronchi were taken from an area as far as possible from the tumor and were dissected free of parenchyma. They were placed in oxygenated Krebs-Henseleit solution (mM: 119 NaCl, 5.4 KCl, 2.5 CaCl\(_2\), 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 25 NaHCO\(_3\), 11.7 glucose) and stored overnight at 4°C. Previous experiences and published data have demonstrated that overnight storage of tissue does not alter its reactivity (8). After removal of adhering fat and connective tissues, rings (5-7 mm length × 1-2 mm internal diameter) of the same bronchus were prepared. The bronchial rings were suspended on tissue hooks in 5-ml organ baths containing Krebs-Henseleit solution, gassed with 95% O\(_2\)-5% CO\(_2\), and maintained at 37°C (pH 7.4). Each preparation was connected to a force displacement transducer (Statham UF-1), and isometric tension changes were recorded in a computer (acquisition system: EMKA France, Bourre, France). Experiments were conducted on parallel groups of rings (6–16, depending on the size of the lung sample). At the end of the pharmacological experiments, rings were fixed and embedded in paraffin for subsequent histological analyses.

**Design of pharmacological experiments.** Previous investigators have suggested that NOS inhibition does not modify ACh-induced contraction of human bronchi, at least in terms of maximal tension development (35). We designed our study to evaluate whether both muscle response to preconditioning protocol (resting tension, in mg) and subsequent response to ACh, in terms of asymptotic maximum, sensitivity, and reactivity (see below), are modified by inhibition of the NOS/arginase balance, which may constitute more subtle phenomena that have not been investigated.

Drug activity was quantified by dose-response curves (DRC) to ACh, which were characterized by the parameters of threshold (sensitivity = −logEC\(_{10}\)), asymptotic maximum (in mg), and slope (reactivity) (Fig. 1). The sensitivity (−logEC\(_{10}\) value) was defined by the negative logarithm of the concentration of ACh that induced a contraction equal to 10% of the maximal effect induced by ACh (10\(^{-3}\) M) (18). In functional studies, slope (and resulting location parameter EC\(_{50}\)) should not be described because it is the result of different transducer functions (each of these having their own DRC). The transducer mechanisms can completely dictate the shape of the DRC.

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**Fig. 1.** Diagram of pharmacological experiments. *Left*: raw data. *Right*: calculated parameters. ACh, acetylcholine; DRC, dose-response curve.
However, intuitively, differences in the slope for the DRC in the same tissue might be used as evidence for differences in reactivity as used during bronchial challenge testing with methacholine inhalation.

The choice of the concentration of inhibitors was based on their concentrations required to inhibit enzyme activity by 50%. At least three concentrations of each specific inhibitor were tested in preliminary experiments: non-NOHA (inhibitor of arginases): 0.1, 1, 10, 100 μM; BEC (inhibitor of arginases): 0.4, 4, 40 μM; Nω-propyl-l-arginine (NOS1 inhibitor): 0.25, 2.5, 25 μM; 1400W (NOS2 inhibitor): 5, 50, 500 nM. The concentration that was finally used was the lowest concentration giving reproducible results. The l-NAME (NOS inhibitor) concentration was that used in previous experiments (10⁻³ M).

The preparations were equilibrated for 90 min with changes in fresh Krebs-Henseleit solution every 15 min before the start of each experiment. A load of 2,500 mg was maintained throughout the equilibrium period. When required, the epithelium was removed by gentle repeated rubbing of the luminal surface with a cotton-tipped applicator, which was checked by histological analysis as described previously (8).

After the initial loading of 2,500 mg and the subsequent equilibrium period, two or three DRC to ACh (10⁻⁷ to 10⁻³ M) were conducted; inhibitors or l-arginine were always added after the first DRC, 30 min before the second DRC. The parameters obtained from the experiments with an inhibitor were compared with the control condition without inhibitor (see Fig. 1). All conditions were run in duplicate.

Immunohistochemistry of NOSs and arginases. Bronchial serial sections (5 μm thick) from 12 randomly selected patients (5 control, 7 COPD) were mounted on Superfrost Plus slides (Fisher Scientific, Fairlawn, NJ). After paraffin removal and tissue section rehydration in graded ethanol concentrations, endogenous peroxidase activity was blocked by 100% H₂O₂ once for 10 min and nonspecific antibody binding sites were blocked with 20% normal swine serum. Rabbit polyclonal antibodies against NOS3 (sc-654), NOS2 (sc-651), and NOS1 (sc-648), a goat polyclonal antibody to arginase I (sc-18351), and a goat polyclonal antibody to arginase II (sc-18360) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Before application of the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0). The dilutions for the primary antibodies, to expose the antigenic sites, the sections were exposed to a demasking pretreatment with sodium citrate buffer (pH 6.0).

Statistical analysis. All values are expressed as means ± SE. Data were analyzed with Statview 5.0 (SAS Institute). For between-group comparisons (COPD, control) Fisher’s exact test was used for categorical variables and the Mann-Whitney U-test for continuous variables. For condition comparisons (within DRC), continuous variables were compared with the Wilcoxon paired test. Spearman’s rank correlation coefficient was calculated to evaluate correlations between pairs of variables. Statistical significance was defined as P < 0.05.

RESULTS

Characteristics of patients. The clinical and functional characteristics of the 22 patients are described in Table 1 according to their respiratory status, i.e., control or COPD subjects.

Protein expression. Immunohistochemistry for α-actin, NOS1, -2, and -3 and arginases I and II are provided in Figs. 2 and 3. Inasmuch as our aim was to further evaluate bronchial reactivity, semiquantitative analysis of NOS expression by bronchial smooth muscle cells was performed, the results of which are provided in Fig. 4 (top). A significantly increased staining for NOS2 is evidenced in patients with COPD compared with control subjects. FEV₁ and NOS2 expression were negatively correlated (r = −0.54, P = 0.027) (Fig. 4, bottom). A negative relationship was evidenced between NOS3 and α-actin expressions (r = −0.42, P = 0.012). A weak expression of both arginases was evidenced in bronchial smooth muscle cells that was not significantly different between COPD and control.

Table 1. Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control (n = 7)</th>
<th>COPD (n = 15)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, F/M</td>
<td>5/2</td>
<td>4/11</td>
<td>0.074</td>
</tr>
<tr>
<td>Age, yr</td>
<td>57±6</td>
<td>59±3</td>
<td>0.75</td>
</tr>
<tr>
<td>Tobacco, pack-yr</td>
<td>3±2</td>
<td>38±5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV₁, % predicted</td>
<td>112±6</td>
<td>86±5</td>
<td>0.004</td>
</tr>
<tr>
<td>FEV₁/FVC, %</td>
<td>82±2</td>
<td>75±1</td>
<td>0.031</td>
</tr>
<tr>
<td>GOLD-D, n</td>
<td>9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>GOLD-1, n</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>GOLD-2-A, n</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Parameter values are means ± SE for n subjects. COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.
Immunoblot analyses of bronchial tissue demonstrated enhanced NOS2 expression in bronchial rings of COPD patients compared with control patients (Fig. 5), whereas expressions of the two other NOSs and arginases were not significantly different between COPD and control patients.

Reproducibility of dose-response curves. Table 2 shows the values of tension (maximum and resting) obtained in the two or three repeated DRC that were obtained in 22 patients (2 DRC) and 10 patients (3 DRC). A small but significant (Wilcoxon test) decrease in both resting and maximum tensions was evidenced after each DRC with ACh: resting tension DRC2 vs. DRC1 ($P = 0.0001$), DRC3 vs. DRC2 ($P = 0.007$); maximum tension DRC2 vs. DRC1 ($P = 0.009$), DRC3 vs. DRC2 ($P = 0.005$). The active contraction (maximum minus resting tension) decreased after the first DRC with ACh (DRC1 vs. DRC2, $P = 0.011$) [this effect has been observed previously (36)] and was subsequently maintained between DRC2 and DRC3 ($P = 0.80$). Consequently, the stability of the ring preparation is obtained after the first DRC.

When L-arginine ($10^{-3}$ M) was added, the decrease in resting tension between DRC2 and DRC3 was not observed ($P = 0.44, n = 9$) while the decrease in maximum tension was still present ($P = 0.011, n = 9$); thus L-arginine inhibited the spontaneous decrease in resting tension (a constrictor response with L-arginine is clearly evidenced in some subjects; data not shown).

When control and COPD subjects are compared, it appears that COPD was associated with a higher resting tension, whereas the response to ACh was similar. A positive relationship was evidenced between the level of resting tension and the expression of NOS2 ($r = 0.61, P = 0.044$).

Effects of inhibitors of NOSs and arginases. Global NOS inhibition with L-NAME induced a decrease in resting tension in both control and COPD patients (Fig. 6, top). This decrease in resting tension with L-NAME was not modified by coincubation with an inhibitor of guanylate cyclase (ODQ, $10^{-5}$ M; data not shown, $n = 3$ experiments). D-NAME had no effect on resting tension (data not shown). Specific NOS2 inhibition led to a lower resting tension in control subjects compared with COPD patients, whereas NOS1 inhibition did not significantly modify resting tension. NOS inhibition did not modify ACh response (sensitivity, reactivity). It must be noted that NOS2 inhibition tended to modify sensitivity and reactivity in the same way as L-NAME, whereas specific NOS1 inhibition tended to act in the opposite direction to L-NAME.

It is clearly evidenced that resting tension can be reduced by L-NAME without affecting maximum tension, which traduces an increase in active contraction [length tension adaptation (3)]. To further determine the mechanisms involved in this unexpected response, an additional experiment evaluated the effect of epithelium removal, which did not modify the effect of L-NAME on resting or maximum tension (the maximum was increased compared with the control condition with epithelium; data not shown).

One inhibitor of arginases (BEC) induced a significant decrease in sensitivity in the whole population, and both inhibitors of arginases induced a decrease in sensitivity in COPD patients compared with control patients (Fig. 6, bottom). Reactivity was not significantly modified by NOS and arginase inhibitors (data not shown).
DISCUSSION

The main result of this study is to suggest that increased NOS2 expression is associated with airflow limitation and is related to ex vivo increased airway tone in COPD patients. The arguments are that NOS2 protein expression is increased in COPD and is related to the degree of airflow limitation and to the degree of resting tension of isolated bronchial rings and that its activity seems involved in a constrictor response to airway stretch. Ex vivo response to ACh was similar in COPD and control subjects; however, inhibition of arginases induced a decrease in sensitivity only in COPD patients, suggesting their activity in this specific condition.

Few studies have evidenced a link between ex vivo mechanical properties of human airways and in vivo findings, namely, the degree of airflow limitation measured by pulmonary function testing. Chemical inhibition or stimulation and its resultant effects can be exquisitely sensitive to the underlying redox state, which is an inherent limitation of our study. However, our results extend those obtained by Tiddens and colleagues (34) with a different methodological approach (isolated small airways inflated and deflated cyclically). They suggested that smooth muscle area and tone were the most important morphological correlates for the dynamic properties of isolated small airways from smokers. For instance, they showed that these airways were characterized by a high intrinsic contractile state suggesting a high smooth muscle tone, and we further extend their results demonstrating increased smooth muscle cell tone in COPD patients. Interestingly, these authors demonstrated that the mechanical characteristics of the first cycle in their experiments were substantially different from the subsequent cycles. On the first cycle, airways were less compliant, had a greater hysteresis, and were less collapsible compared with subsequent cycles. Along this line, we demonstrated differences between the first DRC to acetylcholine and the subsequent response curves (see Reproducibility of dose-response curves). The detailed study of Opazo Saez and
colleagues (23) demonstrated that both force and stress were significantly increased in COPD patients with airflow limitation, and they found a negative correlation between maximal isometric force and FEV1. Consequently, both mechanical properties and pharmacological response of isolated human airways similarly demonstrate the central role of airway muscle tone. The underlying biological pathways responsible for the degree of basal airway tone (or resting tension ex vivo) seem to be partly dependent on NOS2 activity. Resting tension is also related to the activity of 5-lipoxygenase and oxidant metabolites (36).

There are several lines of evidence suggesting that the NOS/arginase balance of the smooth muscle cell may be involved in the regulation of both bronchial remodeling and reactivity. Cyclic stretch induces vascular smooth muscle cell alignment via NO signaling (32). This enzymatic balance regulates both smooth cell proliferation and extracellular matrix deposition, and NO is involved in smooth cell relaxation via cGMP activity. This latter evidence is probably a shortcut inasmuch as the different NOS isoforms have been demonstrated to exert opposite effects in other contractile cell types. For instance, NOS1 promotes myocyte contractility via NO effects on Ca2+ channels (non-cGMP-dependent effect) (5).

Furthermore, on the opposite of vascular wall, epithelial cell-derived NO does not seem to affect bronchial cell muscle contractility (a close physical relationship does not exist for these cells), arguing for the need for a comprehensive view of this enzymatic balance in these cells.

A detailed analysis of histological changes of small airways in COPD has been provided by Hogg and colleagues (16). They demonstrated the absence of increase in volume of smooth muscle in COPD patients classified in GOLD-0, -1, or -2 stages. Consequently, our results with α-actin staining are in agreement. The negative correlation between NOS3 and α-actin expression may be related to the antiproliferative effect of constitutive NO production (17). We suggest an expression of all NOS isoforms by airway smooth muscle cells. Conflicting data exist concerning NOS expression by human airway smooth muscle cells. Initially, Patel and colleagues (25) showed exclusive NOS1 expression, whereas more recently Ricciardolo and coauthors (28) suggested NOS2 expression by airway smooth muscle cells. A detailed analysis of the three NOS protein expressions has been provided for human vascular smooth muscle cells (7). Arginase I expression by vascular smooth muscle cells is well documented, and we further suggest its expression by bronchial smooth muscle cells. Increased expression of arginase I alone is sufficient to increase the proliferation rates of vascular smooth muscle (37). Argi-

Fig. 4. Quantification of NOS immunostaining in bronchial smooth muscle cells. Top: semiquantitative quantification of NOS protein expression (NOS1, -2, -3) in smooth muscle cells (SMC) was done by 2 independent observers blinded for the condition (COPD, control). AU, arbitrary units; NS, not significant. Bottom: a significant correlation was evidenced between forced expiratory volume in 1 s (FEV1) and NOS2 expression.

Fig. 5. Immunoblots for NOSs and arginases and their quantitative analysis. The ratio of each protein over β-actin expression was calculated. Quantification of immunoblots demonstrated a significant increase in NOS2 expression in COPD compared with control patients. The 2 bands that are evidenced for both NOS2 and NOS3 were taken into account since several specific bands can be expressed depending on both cytokine stimulation and proteasome degradation (18a). Top: representative immunoblot of 2 COPD and 2 control patients. MM, molecular mass. Bottom: results of densitometry analysis for 12 patients (7 COPD, 5 controls).
Table 2. Repeated dose-response curves with acetylcholine

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Whole Population (n = 22)</th>
<th>Control (n = 7)</th>
<th>COPD (n = 15)</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>First DRC, n</td>
<td>22</td>
<td>7</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Resting tension, mg</td>
<td>2.688 ± 114</td>
<td>2.499 ± 254</td>
<td>2.777 ± 118</td>
<td>0.34</td>
</tr>
<tr>
<td>Maximum tension, mg</td>
<td>4.467 ± 188</td>
<td>4.114 ± 353</td>
<td>4.632 ± 217</td>
<td>0.31</td>
</tr>
<tr>
<td>Second DRC, n</td>
<td>22</td>
<td>7</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Resting tension, mg</td>
<td>2.030 ± 140</td>
<td>1.574 ± 218</td>
<td>2.243 ± 154</td>
<td>0.03</td>
</tr>
<tr>
<td>Maximum tension, mg</td>
<td>4.134 ± 250</td>
<td>3.453 ± 322</td>
<td>4.451 ± 308</td>
<td>0.06</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>7.17 ± 0.11</td>
<td>7.22 ± 0.29</td>
<td>7.15 ± 0.11</td>
<td>0.75</td>
</tr>
<tr>
<td>Reactivity</td>
<td>33 ± 2</td>
<td>32 ± 3</td>
<td>33 ± 2</td>
<td>0.94</td>
</tr>
<tr>
<td>Third DRC, n</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Resting tension, mg</td>
<td>1.981 ± 204</td>
<td>1.349 ± 77</td>
<td>2.139 ± 221</td>
<td>0.04</td>
</tr>
<tr>
<td>Maximum tension, mg</td>
<td>3.896 ± 379</td>
<td>2.906 ± 633</td>
<td>4.144 ± 416</td>
<td>0.09</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>7.20 ± 0.11</td>
<td>7.06 ± 0.16</td>
<td>7.23 ± 0.14</td>
<td>0.60</td>
</tr>
<tr>
<td>Reactivity</td>
<td>32 ± 2</td>
<td>35 ± 5</td>
<td>32 ± 2</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Parameter values are means ± SE for n subjects. Active tension (maximum tension – resting tension) was similar between control and COPD subjects for the 3 dose-response curves (DRC) (P = 0.75, P = 0.65, P = 0.21, respectively); see the diagram in Fig. 1. *Between control and COPD subjects by Mann-Whitney U-test; significant values are in bold.

Arginase II is located in the mitochondrial matrix and expressed at low levels in many tissues, including lung. Both arginase I and II are oxygen-regulated genes, which may be significant to COPD pathogenesis. Although arginase I expression is upregulated in rodent cells and lung tissue by hypoxia, arginase II is suppressed by hypoxia (38). An upregulation of both arginases has been demonstrated in different models of lung injury (4). Interestingly, immunoreactivity of arginase II has been located in myofibroblasts in bleomycin-induced fibrosis and in the peribronchial connective tissue in hyperoxia-induced injury (10, 27). Because of the low levels of arginase expression we were unable to show significant differences of protein expression between COPD and control subjects, which may also be explained by the fact that all patients had mild COPD and a similar degree of smooth muscle cell staining.

To our best knowledge, extensive data of the effects of NOS inhibition on in vitro response to ACh of human bronchial tissue have not been reported, suggesting that previous investigators have shown little or no effect in this setting. Along this line, Ward and colleagues (35) reported in their study that 1-NAME did not significantly affect either the maximum response to ACh or the EC50. Our results are in agreement, inasmuch as ACh response was unmodified by NOS inhibition. The unexpected dilating effect of 1-NAME on resting tension has not been reported by other investigators under similar experimental conditions. This effect is related to a cGMP-independent effect of NOS2. Along this line, after administration of 1-NAME, the airways of anesthetized and ventilated dogs dilated significantly (6). Furthermore, Grasemann and colleagues (14) recently showed a decrease in FEV1 in healthy

Fig. 6. Effects of NOS and arginase inhibitors on bronchial reactivity. N-propyl-L-arginine (N-propyl) is a specific NOS1 inhibitor, 1400W is a specific NOS2 inhibitor, and Nω-nitro-L-arginine methyl ester (L-NAME) is a nonspecific NOS inhibitor; Nω-hydroxy-nor-L-arginine (nor-NOHA) and (S)-(2-borono-ethyl)-L-cysteine (BEC) are specific inhibitors of arginases. *Between control and COPD subjects by Mann-Whitney U-test; #between control condition (DRC2) and condition with inhibitor by Wilcoxon test. Similar results were obtained with 10 μM nor-NOHA (data not shown). Comparisons between conditions (with and without inhibitor) are provided for the whole group; comparisons between the 2 subgroups (control and COPD) for a given condition are also shown. Results of the statistical comparisons between conditions (with and without inhibitor) for the subgroups (either control or COPD) were as follows: L-NAME induced a significant decrease in resting tension in both COPD (P = 0.046, Wilcoxon test) and control (P = 0.039, Wilcoxon test) subjects. The significance of the inhibitors of arginases on bronchial sensitivity was due to the subgroup of COPD patients. A significant decrease in sensitivity was observed with both nor-NOHA (P = 0.023, Wilcoxon test) and BEC (P = 0.019, Wilcoxon test) compared with the control condition (sensitivity of DRC2).
subjects after L-arginine inhalation despite an increase in exhaled NO, suggesting a constrictor effect of the NOS pathway, which was lost in patients with cystic fibrosis, a condition known to be associated with reduction of NOS2 epithelial expression. Ansarin and colleagues (1) demonstrated that among patients with COPD exhaled NO was inversely correlated with FEV1, further suggesting a relationship between NO production and bronchoconstriction. Overall, recent studies have highlighted the more complex picture of the NOS system in the regulation of bronchomotor tone. Our results demonstrate that COPD patients are characterized by an enhanced response to resting tension (enhanced stretch response) related to the degree of NOS2 expression, which may participate in airflow limitation. The ex vivo increase in airway tone due to basal stretch could seem consistent with the well-described constrictor effect of deep inhalation in COPD patients without overt airflow limitation. According to a theoretical analysis provided by Froeb and Mead (13), the effect of deep inhalation on airway caliber depends on the relative hystereses of airways and lung parenchyma. Fredberg and colleagues (12) found evidence suggesting that mechanical unloading (due to the loss of alveolar attachments) would favor the development of a force maintenance, latch state of smooth muscle. We hypothesize that COPD is characterized by enhanced smooth muscle cell NOS2 expression, which is responsible for a cGMP-independent constrictive effect under basal conditions and a trend toward NOS3 inhibition. This phenotype should be similar to that described by Ricciardolo and colleagues (31) for asthmatic patients. The detrimental effect of NOS2 may be related to protein nitration. Along this line, Maestrelli and coauthors (20) demonstrated that the rate of protein nitration in lung tissue of COPD patients was directly related to NOS2 expression and associated with lower values of FEV1/FVC. Sugiura and colleagues (33) further demonstrated that treatment of COPD patients with steroids resulted in a significant reduction in both nitrotyrosine and NOS2 immunoreactivity in sputum cells compared with pretreatment levels, and that these reductions were correlated with the improvement in FEV1 and airway responsiveness to histamine.

The other original finding is the decrease in sensitivity that is related to arginase inhibition in COPD patients, suggesting that the enhancement of NO bioavailability should be bronchoprotective against cholinergic tone in this subset of patients. This finding is particularly relevant since COPD patients have an increased basal cholinergic bronchomotor tone, and since compounds that release NO are currently used in bronchial diseases (19). It also emphasizes the complexity of airway tone regulation by the NOS isoforms, probably due to both cGMP-dependent and non-cGMP-dependent mechanisms, and due to differential L-arginine bioavailability in subcellular compartments, as suggested for cardiomyocytes (5). Modulation of cholinergic airway reactivity and NO production by endogenous arginase activity has been demonstrated by Meurs and colleagues (21) in guinea pigs. The same group (22) subsequently demonstrated that increased arginase activity underlies allergen-induced deficiency of constitutive NOS-derived NO and airway hyperresponsiveness in guinea pigs, and the study of Zimmermann et al. (39) emphasized the potential role of increased arginase I and II expression in asthma.

It must be acknowledged that our study has several potential limitations. Few patients have been included, representing a narrow spectrum of airflow limitation. Second, all the samples were obtained in a postoperative setting, which may introduce a systematic bias since exhaled NO and NOS activity have been shown to decrease after surgical procedures (2). Third, cellular experiments on isolated airway smooth muscle cells were not performed inasmuch as it was beyond the scope of this pharmacological study, and we were not able to show an increase in arginase expression in COPD. Finally, the cellular mechanism underlying the constrictor effect of NOS2 remains to be determined; it does not seem to involve epithelium release of a constrictor mediator or oxidant synthesis that could have induced NOS uncoupling (40).

In conclusion, COPD is characterized by an increase in NOS2 protein expression that is associated with the degree of airflow limitation and with the degree of resting tension of isolated bronchial rings, and NOS2 activity seems to be involved in a constrictor response to airway stretch. These findings may suggest that NOS2 inhibition, with either a specific inhibitor or corticosteroid, would be an interesting therapeutic approach in patients with the early stage of COPD.

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