Endothelial dysfunction in pulmonary arteries of patients with mild COPD

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Peinado, Víctor I., Joan A. Barberà, Josep Ramírez, Federico P. Gómez, Josep Roca, Lluís J overt, Josep M. Gimferrer, and Robert Rodriguez-Roisin. Endothelial dysfunction in pulmonary arteries of patients with mild COPD. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L908–L913, 1998.—To investigate whether endothelial dysfunction of pulmonary arteries (PA) is present in patients with mild chronic obstructive pulmonary disease (COPD) and to what extent it is related to the morphological abnormalities of PA, we studied 41 patients who underwent lung resection. Patients were divided into the following groups: nonsmokers (n = 7), smokers with normal lung function (n = 13), and COPD (n = 21). Endothelium-dependent relaxation mediated by nitric oxide was evaluated in vitro in PA rings exposed to cumulative concentrations of acetylcholine (ACh) and ADP. Structural abnormalities of PA were assessed morphometrically. PA of COPD patients developed lower maximal relaxation in response to ADP than both nonsmokers and smokers (P < 0.05 each) and a trend to reduced relaxation in response to ACh (P = 0.08). Maximal relaxation to ADP correlated with the degree of airflow obstruction (r = 0.48, P < 0.01). Morphometrical analysis of PA revealed thicker intimas, especially in small arteries, in both smokers and COPD compared with nonsmokers (P < 0.05 each). We conclude that endothelial dysfunction of PA is already present in patients with mild COPD. In these patients, as well as in smokers with normal lung function, small arteries show thickened intimas, suggesting that tobacco consumption may play a critical role in the pathogenesis of pulmonary vascular abnormalities in COPD.

endothelium; intimal layer; nitric oxide; pulmonary circulation; tobacco smoking

ENDOTHELIUM-DERIVED nitric oxide (NO) is a potent vasodilator that plays an important role in modulating pulmonary vascular tone (14). Impairment of endothelium-dependent vascular relaxation has been shown in pulmonary arteries of patients with end-stage obstructive lung diseases (namely, bronchiectasis and emphysema) who underwent lung transplantation (15). In that study, the degree of impairment of vascular relaxation was correlated with both the severity of hypoxemia and the thickening of the intimal layer of pulmonary arteries. Because hypoxia may attenuate the endothelium-dependent vascular relaxation (8), it was postulated that chronic hypoxemia might account for the diminished release of endothelium-derived relaxing factors (15). Moreover, reduced expression of NO synthase (NOS) has been shown in pulmonary arteries of patients with pulmonary hypertension, the immunoreactivity to NOS being inversely related to the structural derangement of pulmonary arteries (17). Accordingly, in pulmonary arteries of patients with advanced obstructive lung disorders with secondary pulmonary hypertension, the development of structural abnormalities may be accompanied by an impaired release of endothelium-derived NO, which in turn may further enhance the progression of pulmonary hypertension.

In a previous study, we showed that patients with mild chronic obstructive pulmonary disease (COPD) may exhibit intimal thickening in small pulmonary muscular arteries and that the degree of intimal thickening was associated with a reduced reactivity to hypoxic stimulus (4). Accordingly, we hypothesized that endothelial dysfunction might be already present in pulmonary arteries of patients with mild COPD. Because these patients usually do not exhibit severe hypoxemia, we postulated that, in mild COPD, the diminished release of endothelium-derived relaxing factors (namely, NO) could be related to the underlying structural derangement of pulmonary arteries.

The present study was therefore designed to assess in vitro the reactivity of pulmonary artery rings to NO-dependent and NO-independent vasodilators and to evaluate morphologically the abnormalities of pulmonary muscular arteries in patients with mild COPD and in control subjects who underwent lung resective surgery for lung carcinoma.

MATERIALS AND METHODS

Subjects. Forty-one patients (32 males and 9 females) who underwent lobectomy or pneumonectomy because of lung carcinoma were studied. Pulmonary function tests (forced spirometry, lung volumes, carbon monoxide diffusing capacity, and blood gas analysis) were performed in the days preceding surgery, as previously described (4).

On the basis of smoking history and the results of forced spirometry, patients were divided into the following three groups: 1) nonsmokers, all of whom had normal lung function; 2) smokers with normal lung function; and 3) COPD, smokers with airflow obstruction, as defined by a forced expiratory volume in 1 s (FEV1)-to-forced vital capacity (FVC) ratio lower than 70%. General characteristics and lung function measurements of each group of patients are shown in Table 1.

In vitro assessment of vascular reactivity. Resected lung specimens were placed in cold Krebs-Henseleit buffer (in mM: 118 NaCl, 24 NaHCO3, 11.1 glucose, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, and 1.6 CaCl2) gassed with 95% O2-5% CO2 (pH = 7.35–7.45). Arterial segments with an external diameter of 1.5–2.5 mm were carefully dissected free of visible fat and...
Table 1. General characteristics and lung function measurements

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>COPD</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>13</td>
<td>21</td>
</tr>
<tr>
<td>Age, yr</td>
<td>62 ± 11</td>
<td>57 ± 10</td>
<td>63 ± 11</td>
</tr>
<tr>
<td>FEV₁, %predicted</td>
<td>96 ± 14</td>
<td>97 ± 16</td>
<td>72 ± 15†</td>
</tr>
<tr>
<td>FVC, %predicted</td>
<td>85 ± 15</td>
<td>95 ± 15</td>
<td>84 ± 14</td>
</tr>
<tr>
<td>FEV₁/FVC, %</td>
<td>85 ± 5</td>
<td>76 ± 5</td>
<td>60 ± 7†</td>
</tr>
<tr>
<td>FEF25–75, %predicted</td>
<td>126 ± 27</td>
<td>90 ± 27</td>
<td>42 ± 15†</td>
</tr>
<tr>
<td>TLC, %predicted</td>
<td>92 ± 7</td>
<td>100 ± 17</td>
<td>108 ± 16</td>
</tr>
<tr>
<td>RV, %predicted</td>
<td>103 ± 31</td>
<td>115 ± 34</td>
<td>146 ± 37†</td>
</tr>
<tr>
<td>DCO₂, %predicted</td>
<td>82 ± 14</td>
<td>84 ± 23</td>
<td>90 ± 23</td>
</tr>
<tr>
<td>PaO₂, mmHg</td>
<td>96 ± 14</td>
<td>87 ± 10</td>
<td>83 ± 4</td>
</tr>
<tr>
<td>PaCO₂, mmHg</td>
<td>32 ± 8</td>
<td>36 ± 3</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>a-APo₂, mmHg</td>
<td>14 ± 9</td>
<td>18 ± 8</td>
<td>24 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± SD. COPD, chronic obstructive pulmonary disease; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; FEF25–75, forced midexpiratory flow; TLC, total lung capacity; RV, residual volume; DCO₂, carbon monoxide diffusing capacity; PaO₂, partial pressure of arterial oxygen; PaCO₂, partial pressure of arterial carbon dioxide; a-APo₂, alveolar-arterial oxygen pressure difference. *P < 0.05 vs. nonsmokers. †P < 0.05 vs. smokers.

connective tissue and cut into rings ~2 mm long. Four rings of each subject were suspended as cylindrical segments between stainless steel hooks in 10-ml organ bath chambers (Letica, Barcelona, Spain) filled with Krebs-Henseleit buffer, aerated continuously with 95% O₂-5% CO₂ and kept at 37°C by an outer water bath warmed by a recirculating heater. Changes in isometric tension were recorded in each ring by a force transducer (Letica) connected to a chart drive recorder. A resting tension of 1.75 g was applied to each ring, according to preliminary length-tension studies. The rings were allowed to equilibrate for at least 90 min, during which tension was readjusted and the bath fluid was changed every 15 min. After equilibration, all rings were preincubated during 30 min with indomethacin (10⁻⁵ M), which was present in the bath throughout all the experiments to inhibit the synthesis of cyclooxygenase products of arachidonic acid.

All rings were submaximally precontracted with L-phenylephrine dichloride (PE; 10⁻⁶ to 10⁻⁵ M) to obtain a stable plateau of tension. In the majority of experiments, two rings were tested to cumulative concentrations of acetylcholine (ACh; 10⁻¹⁰ to 10⁻⁴ M) and one ring to ADP (10⁻⁴ to 10⁻⁴ M), both endothelium-dependent vasodilators. Relaxation to cumulative doses of the endothelium-independent vasodilator sodium nitroprusside (SNP; 10⁻⁴ to 10⁻⁴ M) was also studied in an additional ring from all patients. In a second set of experiments, the same vasodilators were assayed in the presence of N⁶-monomethyl-L-arginine (L-NAME; 10⁻⁴ M), an inhibitor of NOS. All drugs were purchased from Sigma Chemical (St. Louis, MO).

Relaxation of each pulmonary artery ring was determined by measuring the reduction in tone in response to a cumulative dose of the vasodilating agent and expressed as the percent reduction from the value recorded after precontraction with PE. Maximal relaxation was the greatest reduction in tone in response to a vasodilator. The concentration needed to reach 50% of maximal relaxation (EC₅₀) was determined by curve fitting and expressed as its negative logarithm. Moreover, the area under the curve in each dose-response study was calculated using the algorithm of the statistical package SigmaStat (Jandel, Erkrath, Germany).

Because an insufficient source of endogenous L-arginine might potentially account for the impairment of endothelium-dependent pulmonary artery relaxation (8), in an additional set of experiments, pulmonary artery rings from five patients were assayed to ADP and ACh as described before, with the exception that L-arginine (Merck, Darmstadt, Germany) was present in two of the four chambers throughout all experiments.

The presence of endothelium in each pulmonary artery ring was confirmed at the end of each experiment by specific staining with antisera to von Willebrand factor (factor VIII-related antigen; Dako, Santa Barbara, CA).

Morphometric studies. Pulmonary muscular arteries were analyzed in Formalin-fixed paraffin-embedded lung tissue sections processed with elastic orcein stain. All arteries with an external diameter <1 mm and with complete elastic lamina were evaluated using a computerized image-analysis system (Microm, Barcelona, Spain) as previously described (4). Briefly, the external diameter was measured as the widest distance between external elastic lamina, perpendicular to the greatest longitudinal axis of each artery. External and internal elastic laminae and the inner aspect of the intima were outlined, and the area occupied by the muscular layer, the intimal layer, and the lumen was computed. Results were expressed as percentage of total area.

Statistics. All data are expressed throughout as means ± SD. Mean values of variables of interest from the three groups were compared by means of one-way analysis of variance, and post hoc pairwise comparisons using the Student-Newman-Keuls test were applied when the F-value indicated significant differences among group means. The pharmacological responses of the rings treated and untreated with L-arginine from the same patient were compared using the Wilcoxon signed rank test. For the comparisons of morphological variables between groups, measurements in each subject were averaged. Relationships between variables were assessed using the Pearson’s correlation test. A probability value of P < 0.05 was considered significant in all cases.

Because morphological abnormalities of pulmonary arteries may not affect uniformly vessels of different sizes and because the number and the sizes of the arteries that were evaluated on each patient were different, the relationships between the size of lumen, intima, media and artery wall and the artery diameter were determined for each subject. The average relationship of each group was then estimated through a random effects regression analysis using the restricted maximum likelihood method described by Feldman (16). This analysis allows the estimation of group regression lines, as well as confidence intervals, for a specific relationship, weighing each patient’s data and minimizing the within-subject and the between-subject variability in each group. The differences between regressions in the three groups were evaluated using x² statistics. More details on such analysis and its appropriateness for the evaluation of lung morphological measurements have been described elsewhere (4, 6).

RESULTS

Lung function measurements from the three groups are shown in Table 1. Patients in the COPD group showed moderate airflow obstruction (FEV₁, 72 ± 15% of predicted) and gas trapping compared with the other groups. COPD patients also showed lower arterial partial O₂ pressure (PaO₂) than nonsmokers, although it was >80 mmHg in the majority of patients.

Measurements of vascular reactivity. The results of reactivity studies in pulmonary artery rings from the three groups are summarized in Table 2. Maximal contraction to PE was similar in the three groups.
Table 2. Vascular responses of pulmonary artery rings

<table>
<thead>
<tr>
<th>Maximal relaxation, %</th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>COPD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction to PE (10^{-5} M), mg</td>
<td>997 ± 275</td>
<td>929 ± 226</td>
<td>1,035 ± 382</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Values are means ± SD. PE, L-phenylephrine; SNP, sodium nitroprusside; L-NAME, N^6-monomethyl-L-arginine; EC_{50}, concentration needed to reach 50% of maximal relaxation. P values determined using ANOVA. * P < 0.05 vs. nonsmokers. † P < 0.05 vs. smokers.

Compared with both nonsmokers and smokers, there was a significant reduction in the maximal relaxation in response to ADP in pulmonary artery rings in the COPD group (Table 2 and Fig. 1). Maximal relaxation to ACh was not significantly reduced in the mild COPD group (Table 2). However, the area under the dose-response curve to ACh, which summarizes the response at the different concentrations (Fig. 1), was greater in COPD than in nonsmokers (499 ± 116 and 385 ± 69, respectively, P < 0.05). No differences in the EC_{50} of either ACh or ADP dose-response studies were shown among the different groups. When artery rings were treated with SNP, all of the rings relaxed maximally, and no differences between groups were observed.

The decrease in vascular reactivity of arteries from COPD patients was not due to endothelial damage during manipulation, since the histological evaluation with factor VIII confirmed the presence of endothelial cells on the luminal side of the arterial wall. Moreover, when rings were exposed to the competitive inhibitor of NOS, L-NAME, relaxation induced by both ACh and ADP was almost abolished (Table 2), indicating that both vasodilators operated through the l-arginine-NO pathway.

The maximal relaxations induced by both ACh and ADP correlated with the FEV_{1}-to-FVC ratio (r = 0.48, P < 0.01 and r = 0.38, P < 0.05, respectively; Fig. 2). Moreover, maximal relaxation induced by ADP was inversely correlated with the alveolar-arterial O2 pressure difference (r = -0.51, P < 0.01).

No differences in maximal relaxation to ACh and ADP were observed when arterial rings were incubated with and without L-arginine (P = 0.13 and P = 0.44, respectively), thus indicating that the diminished response to these agents was not due to a substrate deficiency.

Morphological evaluation. Morphometric measurements in pulmonary muscular arteries from the three groups of patients are shown in Table 3. The number of arteries analyzed in each subject was similar in the three groups. Likewise, there was a similar distribution in the external diameter and in the degree of narrowing of the arteries that were examined among the groups.

Both COPD patients and smokers showed thicker intimas than nonsmokers (Table 3). By contrast, no differences in the size of the muscular layer were shown. The degree of intimal thickening in COPD patients and smokers was similar.

Estimated parameters of the relationship between thickness of the intimal layer and calculated artery diameter disclosed differences between nonsmokers and both smokers and COPD (P = 0.033 and P = 0.003, respectively) that were significant in arteries with small diameters (<182 µm, nonsmokers vs. smokers; and <220 µm, nonsmokers vs. COPD). No differences...
between smokers and COPD were shown in the relationship between intimal thickness and artery diameter. Furthermore, the analysis of muscular size as a function of artery diameter showed no differences among the three groups.

**DISCUSSION**

The results of the present study show impaired relaxation to the NO-dependent vasodilator ADP in pulmonary arteries of patients with mild COPD, which was associated with enlargement of the intimal layer in small pulmonary muscular arteries. Moreover, in smokers with normal lung function, pulmonary muscular arteries also exhibited intimal thickening, although the NO-dependent relaxation did not differ from that in nonsmokers.

In our study, maximal relaxation of pulmonary artery rings induced by ADP was diminished in COPD patients. Relaxation induced by ACh was also diminished in the COPD group, as shown by a greater area under the dose-response curve, although its magnitude was lower than that shown with ADP (Table 2). This different response to ADP and to ACh might be related to the variable effects that ACh may exert on pulmonary arteries, where it may induce contraction when used at high concentrations (2). Contrasting with the response to NO-dependent vasodilators, relaxation induced by a direct NO donor, such as SNP, was maximal in the three study groups. Moreover, inhibition of NO synthesis with L-NAME practically abolished the response to NO-dependent vasodilators. Overall, these results are consistent with endothelial dysfunction in pulmonary arteries of patients with mild COPD.

Dinh-Xuan and co-workers (15) showed endothelial dysfunction in pulmonary arteries of patients with end-stage obstructive lung diseases (bronchiectasis and emphysema). Results of the present study are in agreement with this finding and extend it to the initial stage of the COPD spectrum. Accordingly, endothelial dysfunction of pulmonary arteries appears not to be a phenomenon restricted to advanced COPD. Yet, it might be initiated early on in the course of the disease when airflow obstruction is moderate and PaO₂ is within the normal range. Results of the present study also support our previous suggestion that the impairment of hypoxic pulmonary vasoconstriction in patients with mild COPD is more likely due to the functional impairment of endothelium-dependent relaxation than to the stiffness of the pulmonary artery wall produced by connective tissue proliferation. The correlation between maximal relaxation to both ACh and ADP and the severity of airflow obstruction (Fig. 2) suggest that endothelial dysfunction may be enhanced with disease progression. Interestingly, pulmonary arteries of smokers with normal lung function also showed a trend to lower relaxation in response to NO-dependent vasodilators (Table 2), although it was not significantly different from that in nonsmokers. This raises the possibility that endothelial impairment might originate even before airflow obstruction is apparent. In this regard, it is interesting to note that impairment of endothelium-dependent arterial dilatation of systemic arteries has been shown in both active and passive smokers (10, 24).

Table 3. Morphometric measurements on pulmonary muscular arteries

<table>
<thead>
<tr>
<th>Arteries/patient</th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>COPD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured external diameter, µm</td>
<td>136±70</td>
<td>133±47</td>
<td>112±22</td>
<td>0.27</td>
</tr>
<tr>
<td>Calculated external diameter, µm</td>
<td>242±139</td>
<td>229±67</td>
<td>200±37</td>
<td>0.32</td>
</tr>
<tr>
<td>Index of narrowing, %</td>
<td>54±5</td>
<td>56±7</td>
<td>55±8</td>
<td>0.79</td>
</tr>
<tr>
<td>Total area, mm²·10⁻⁴</td>
<td>291±299</td>
<td>244±151</td>
<td>184±94</td>
<td>0.28</td>
</tr>
<tr>
<td>Wall thickness, %measured radius</td>
<td>40.8±7.9</td>
<td>47.9±9.0</td>
<td>48.7±8.1</td>
<td>0.10</td>
</tr>
<tr>
<td>Lumen area, %total area</td>
<td>43.3±7.7</td>
<td>36.6±9.3</td>
<td>35.4±7.9</td>
<td>0.10</td>
</tr>
<tr>
<td>Intimal area, %total area</td>
<td>20.9±4.9</td>
<td>27.1±5.6*</td>
<td>28.2±6.0*</td>
<td>0.02</td>
</tr>
<tr>
<td>Muscular area, %total area</td>
<td>35.7±4.5</td>
<td>36.3±7.1</td>
<td>36.5±6.8</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Values are means ± SD. P values determined using ANOVA. Index of narrowing determined by equation 100·[(measured total area/calculated total area)]. *P < 0.05 vs. nonsmokers.
is controversial. Whereas some studies have shown that hypoxia may downregulate NO synthesis (8), others seem to indicate that it may exert the opposite effect (7, 21, 31).

Results of morphometric measurements of pulmonary muscular arteries in the present series are in agreement with data previously reported by our group (4). Compared with nonsmokers, patients with mild COPD showed thickening of the intimal layer of small pulmonary muscular arteries (Table 3). This finding reinforces the notion that structural alterations of the intimal layer in small pulmonary muscular arteries may be an early feature in COPD. Furthermore, in the present study, we have shown intimal thickening in pulmonary arteries of smokers who had normal lung function to a similar degree to that seen in COPD patients (Table 3). This suggests that tobacco consumption might be associated with structural abnormalities of pulmonary vessels, even when airflow obstruction is not apparent. Accordingly, tobacco consumption seems to play a pivotal role in the remodeling process of pulmonary vasculature. MacNee and associates (22) showed that active cigarette smoking delays the transit of neutrophils in the pulmonary circulation and increases their capability to interact with pulmonary endothelium. Indeed, activation of endothelium by inflammatory mediators in pulmonary vessels of smokers has been shown by an increased expression of inducible adhesion molecules (E- and P-selectin and intercellular adhesion molecule-1; see Ref. 18). Endothelial activation may promote the release of cytokines and growth factors (13, 29) that induce the proliferation of fibroblasts and the laying down of cell matrix proteins (9).

Different studies have related the morphological abnormalities of the vessel wall with an impaired release of NO by endothelial cells. Giaid and Saleh (17) demonstrated that, in patients with primary pulmonary hypertension, endothelial NOS (eNOS) immunoreactivity was reduced in those cases in which more severe grades of arteriopathy were exhibited. Furthermore, Dinh-Xuan and colleagues (15) also showed in patients with end-stage COPD an inverse relationship between thickening of the intimal layer and the relaxation induced by ACh. In our study, pulmonary arteries in the COPD group were those that showed the lowest reactivity to NO-dependent vasodilators and the greatest thickening of the intimal layer (Fig. 3). To what extent the diminished release of NO by endothelial cells contributes to the intimal enlargement or is the consequence of a persistent structural damage remains uncertain. NO possesses antiproliferative effects that may suppress both neointimal vascular thickening and angiogenesis (23, 25). In addition, NO inhibition has been shown to increase collagenous protein synthesis (25). However, results of our study show intimal thickening in pulmonary arteries of smokers whose endothelial function did not differ significantly from that of nonsmokers (Table 3 and Fig. 3), suggesting that structural changes might antecede or be independent of the impairment of pulmonary vascular reactivity. Cigarette smoke induces cell proliferation in small vessels (27) and alters endothelium permeability (1), changes that may ensue either from a direct effect of cigarette smoke on pulmonary vasculature or from the release of cytokines by inflammatory cells, which are commonly present in the airways of chronic smokers (3, 5, 12). Because the severity of bronchial inflammation correlates with that of pulmonary vessel abnormalities (4, 19, 30), we hypothesize that the inflammatory process associated with tobacco consumption may play a key role in the pathogenesis of pulmonary vascular abnormalities of COPD. In this scenario, the continuous release of NO by endothelial cells may be relevant in preserving the integrity of the vessel wall under both physiological and pathological conditions, since endothelium-derived NO not only modulates the vascular tone but also regulates the interactions between vascular endothelium and circulating inflammatory cells (11, 20, 26). The latter might be particularly relevant at sites of inflammation, where endothelium must protect itself against toxic mediators released from chemoattractant-stimulated, emigrating neutrophils. If this is the case, pulmonary vascular remodeling in COPD may follow, at least in part, a pathogenesis similar to that proposed in other pulmonary hypertensive diseases (29). Accordingly, it can be hypothesized that the inflammatory process associated with tobacco smoking might be responsible, on the one hand, for the enlargement of the intimal layer through the release of growth factors and, on the other, for the reduced expression of eNOS as a consequence of the action of inflammatory mediators. Reduced NO release can diminish vascular reactivity and, additionally, may further promote and perpetuate the remodeling process of the vascular wall, since NO also possesses antiproliferative properties (28, 32). Further studies characterizing the inflammatory infiltrate and the expression of eNOS in pulmonary arteries of both smokers and patients with mild COPD should contribute to elucidate this hypothesis.

In summary, the present study shows that endothelial dysfunction of pulmonary arteries may be already present in patients with mild COPD. Furthermore, the
observation that smokers with normal lung function exhibited intimal thickening in small pulmonary arteries suggests that tobacco consumption may play a central role in the pathogenesis of the structural and functional alterations of the pulmonary vasculature in COPD.

We thank the surgical staff of the Department of Thoracic Surgery and the technical staff of the Department of Pathology for collaboration and W. J. Jiménez and J. Ros for assistance in the vascular reactivity studies.

This study was supported by Grants FIS 95/0572 and 96/0762 from the Fondo de Investigación Sanitaria, SEPAR-95 from the Sociedad Española de Neumología y Cirugía Torácica, and FUCAP-96 from the Fundación Catalana de Pneumología and an educational grant from Berenguer-Infaire.

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Received 2 December 1997; accepted in final form 26 February 1998.

REFERENCES