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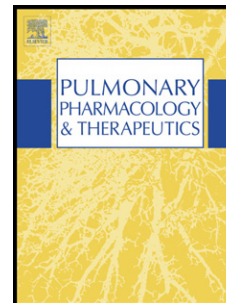
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# Accepted Manuscript



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## Association of ex vivo vascular and bronchial dysfunctions in smokers

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**Short title:** vascular and airway dysfunctions

**Abstract**

Word count: 204

**Background.** It has recently been shown that systemic endothelial dysfunction is associated with airflow limitation in COPD. We conducted this ex vivo study to assess whether endothelial dysfunction of pulmonary arteries of former smokers was associated with modifications of airway functions.

**Methods.** Pharmacological experiments were conducted on arterial and bronchial rings obtained from lung specimen of 20 patients: 13 smokers without COPD and 7 smokers with mild to moderate COPD (GOLD class I or II). The impairment of acetylcholine-mediated vasodilation (constriction) of precontracted arterial rings defined endothelial dysfunction. Resting tone (initial and after a contraction test) and cGMP-mediated dilation of bronchial rings in response to zaprinast were evaluated.

**Results.** Initial airway resting tone was correlated with airflow limitation ( $FEV_1$  % predicted:  $Rho = -0.49$ ;  $p = 0.032$ ). The acetylcholine response of arterial rings was correlated with zaprinast-induced bronchodilation ( $Rho = 0.54$ ,  $p = 0.019$ ). Patients with endothelial dysfunction ( $n = 5$ ), as compared with those displaying no dysfunction ( $n = 15$ ), were characterized by an increased resting tone (after contraction test), an impaired response to zaprinast but a similar degree of airflow limitation ( $FEV_1$ ).

**Conclusion.** Endothelial dysfunction of pulmonary arteries is associated with increased resting tone and impaired cGMP-mediated dilation of airways in former smokers, suggesting common underlying mechanisms of pulmonary arterial and bronchial dysfunctions.

**Key words:** zaprinast, acetylcholine, bronchial ring, arterial ring, endothelial dysfunction

## 1. Introduction

Recent researches on the pathogenesis of chronic obstructive pulmonary disease (COPD) suggest that perturbations in the vasculature may occur early in COPD [1-3]. Nitric oxide (NO)-mediated, endothelium-dependent relaxation provoked by adenosine diphosphate and acetylcholine is attenuated in the excised pulmonary arteries of patients with COPD compared with those of smoking and non-smoking control subjects [4]. Barr and colleagues demonstrated that impaired flow-mediated dilation, assessed by brachial artery flow-mediated dilation, is associated with low pulmonary function and emphysema in ex-smokers [1]. In this latter study, a linear relationship was demonstrated between flow-mediated dilation and post-bronchodilator FEV<sub>1</sub>, and the magnitude of this association was the same in former smokers who had not developed COPD (FEV<sub>1</sub>/FVC ≥ 70%) as in the overall sample (mild to severe COPD). The authors postulated that the systemic nature of endothelial function made likely the alteration of endothelial reactivity in the pulmonary circulation. Based on this assumption, one may hypothesize that endothelial dysfunction of pulmonary arteries would be associated with impaired airway function. Accordingly, Peinado and colleagues showed that maximal relaxation of pulmonary arterial rings induced by acetylcholine is correlated with the FEV<sub>1</sub>/FVC ratio of COPD patients [4]. Inasmuch as endothelial dysfunction is associated with dysregulated nitric oxide (NO) metabolism, we speculated that NO-mediated bronchodilation would also be impaired. We therefore conducted this study to assess whether endothelial dysfunction of pulmonary arteries was associated with modifications of the level of resting tone of isolated airways and their cGMP-mediated dilation. Both parameters are NO-dependent, and the former has previously been suggested to be associated with the level of airflow limitation [5].

## 2. Material and Methods

### 2.1. Patients

Consecutive patients referred for lung surgical procedure (lobectomy or pneumonectomy for lung tumour) were eligible. Since impaired flow-mediated systemic dilation has been associated with low pulmonary function in former smokers, only ex-smokers were included (>15 Pack-Year). Based on the post-bronchodilator value of pre-operative pulmonary function test, patients were classified according to GOLD criteria [6]. All patients were informed of these biological experiments (bioethics' law of august 2004). This study was approved by our Institutional Review Board.

### 2.2. Pharmacological studies

Just after resection, segments of pulmonary arteries and bronchi were taken from an area as far as possible from the tumour and were dissected free of parenchyma. Arterial rings were evaluated immediately after dissection, whereas bronchial rings were placed in oxygenated Krebs's solution and stored overnight at 4°C, as previously described [5, 7, 8].

Intralobar arterial rings (inside diameter of 1.5-2 mm) or bronchial rings (inside diameter 1-2 mm) were suspended on tissue hooks in 5 ml organ baths containing Krebs-Henseleit solution (NaCl, 119 mM; KCl, 5.4 mM; CaCl<sub>2</sub>, 2.5 mM; KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM; MgSO<sub>4</sub>, 1.2 mM; NaHCO<sub>3</sub>, 25 mM; glucose, 11.7 mM), gassed with 95% O<sub>2</sub>: 5% CO<sub>2</sub> and maintained at 37°C (pH 7.4). Each preparation was connected to a force displacement transducer (Statham UF-1) and isometric tension changes recorded in a computer (acquisition system: EMKA France, Bourre, France). All conditions were run in duplicate (when the duplicate experiments show discordant values, no result is given).

#### 2.2.1. Arterial experiments:

Endothelium-dependent relaxation was assessed by measuring the responses to acetylcholine (ACh, Pharmacie Centrale des Hôpitaux, Paris, France) in vessels precontracted with L-phenylephrine dichloride ( $10^{-5}$  M). Relaxation to ACh was expressed as a percentage of relaxation of phenylephrine induced tone. The absence of ACh-induced dilation or even a contractile response defined endothelial dysfunction, which constitutes a stringent definition that is based on the upper normal limit of the response in non smokers with normal lung function in our laboratory. ~~accordingly to previous experiments done in an historical group of non-smokers.~~ We previously demonstrated that the decrease in vascular reactivity was not due to endothelial damage during manipulations [7].

#### 2.2.2. Bronchial experiments:

After the initial loading of 2.5 g and subsequent equilibrium period, an initial resting tension (RT1) was obtained, bronchial viability was assessed using  $10^{-3}$  M ACh. Then a new resting tension (RT2) was obtained, and a dose response curve to zaprinast ( $10^{-8}$  to  $10^{-5}$  M) was conducted. At the end of the experiment, after an equilibrium period to resting tension, maximal dilation ( $10^{-3}$  M theophylline) was assessed, as previously described [9]. The absence of epithelial damage has also been checked in preliminary experiments [5]. Results were expressed as raw values (g) or percentage of theophylline-induced relaxation, as specified. The investigators performing bronchial experiments were blinded for the results of vascular experiments.

#### 2.3. Statistical analyses

Results are expressed as median [interquartile range]. Data were analyzed using Statview 5.0 (SAS Institute, Inc, North Carolina). For intergroup comparisons, categorical variables were compared using the chi-squared test or Fisher's exact test, and continuous variables were compared using the Mann-Whitney U test or Kruskal Wallis test as appropriate. For

intragroup comparisons, continuous variables were compared using the Wilcoxon paired test. The Spearman's rank correlation coefficient was used to examine possible correlation between two variables. A *P* value less than 0.05 was considered as significant.

### 3. Results

The clinical and functional characteristics of the 20 patients who were included are described in table 1. The characteristics of patients with and without dysfunction were similar. The figure 1 shows acetylcholine response of precontracted arterial rings in patients with (n=5) and without endothelial dysfunction (n=15).

The degree of airflow limitation was significantly correlated with the level of initial resting tension (RT1) (Rho= -0.49; p=0.032), and tended to be correlated with theophylline-induced maximal dilation (Rho= -0.38, p=0.096).

The response to acetylcholine (constriction to dilation) of arterial rings was correlated with the degree of zaprinast-induced bronchodilation (figure 2), and tended to be correlated with the FEV<sub>1</sub>/FVC ratio (with ACh 10<sup>-5</sup> M, Rho=0.37, p=0.11).

The figure 3 shows that patients with endothelial dysfunction (n=5), as compared to those without dysfunction (n=15), were characterized by a higher level of resting tone after acetylcholine-induced contraction (RT2) and higher levels of tension under each single concentration of zaprinast (figure 3, upper panel). Since the level of RT2 was significantly different between the two groups of patients, their response to zaprinast was further evaluated as percentage of theophylline-induced maximal dilation (similar for the two groups). The impaired response to zaprinast is further confirmed (figure 3, lower panel), suggesting an impairment of cGMP-mediated dilation in COPD patients with endothelial dysfunction.



## 4. Discussion

### 4.1. Background

It has been shown that an impairment of flow-mediated dilation in systemic circulation occurs early in COPD, and is associated with airflow limitation, independently of smoking history, further suggesting a link between vascular and bronchial functions [1]. We suggest that in former smokers, endothelial dysfunction of pulmonary arteries is associated with impairment of both spontaneous resting tone and cGMP-mediated dilation of airways, establishing a biological link between pulmonary arterial and bronchial functions. To our best knowledge, comparison of arterial and bronchial pharmacological functions obtained from the same lung sample has never been made, and the description of dysfunction of pulmonary arteries has been assessed in a very limited number of studies [4, 10, 11]. A recent pulmonary perspective supported by the National Heart, Lung, and Blood Institute stated that there is a need for methods linking in vitro observations to in vivo disease state with regard to airway smooth muscle function [12]. Along this line we show an inverse relationship between the level of resting tone and FEV<sub>1</sub>, which is consistent with the fact that increased smooth muscle tone may contribute to airflow limitation in COPD, as previously suggested [5, 13, 14]. In our limited series of patients, airflow limitation was similar whether or not endothelial dysfunction was present. Several factors have been shown to contribute to systemic endothelial dysfunction, and our study was not powered to describe clinical and functional factors associated with pulmonary endothelial dysfunction.

### 4.1. Endothelial dysfunction

There is no consensual definition of pulmonary endothelial dysfunction, but we do think that the absence of acetylcholine-induced dilation or even contractile response constitutes a drastic definition. We observed that one quarter of our COPD patients displayed pulmonary endothelial dysfunction, which is in agreement with our subsequent results observed in a larger series [8]. No data are available in the literature for further comparison, but a ~30% incidence of pulmonary hypertension in COPD patients has been reported [10]. In the study of Peinado and colleagues in mild COPD patients (mean  $\pm$  SD:  $24.9 \pm 22.9$  %), the dilator response to acetylcholine was diminished as compared to non smokers ( $40.1 \pm 22.0$  %), without reaching statistical significance. The standard deviations of their results suggest that some COPD patients depicted a constrictor response to acetylcholine while almost all non smokers have a vasodilator response. Obviously, our results need to be confirmed in a larger sample of patients due to the restricted size of our sample with endothelial dysfunction.

#### *4.3. Bronchial dysfunction*

The nil to moderate degree of cGMP-mediated bronchial dilation that is evidenced is consistent with the results of other investigators [15-17], emphasising the confounding effect of underlying pathology (such as endothelial dysfunction) and the modest contribution of cGMP-mediated dilation in airways. The presence of a systemic endothelial dysfunction suggests an impairment of NO-mediated relaxation in COPD. Our results further argue for a generalized impairment of NO-dependent functions, affecting both vascular and bronchial lung compartments. Airway blood flow reactivity has also been shown to be impaired in ex-smokers [18]. Mullershausen and colleagues have demonstrated a similar mechanism of desensitization of NO/cGMP signaling in smooth muscle of both aorta and bronchi of rats, involving phosphodiesterase (PDE) 5 activation [19], giving insight into the underlying

mechanisms of generalized endothelial dysfunction. An increased expression of PDE 5 in models of pulmonary hypertension has been demonstrated [20].

Figure 3 shows that resting tension after ACh was higher in the group with endothelial dysfunction that could already be related to an impaired relaxation after contraction. The bronchial relaxation induced by the nonselective phosphodiesterase (PDE) inhibitor (theophylline) was similar whatever the presence or absence of impaired cGMP-dependent relaxation (see Figure 3). It first suggests that cAMP-dependent relaxation is preserved whatever the degree of impairment of cGMP-mediated relaxation. In human bronchial smooth muscle, the presence of PDE I, II, III, IV (cAMP specific) and V (cGMP specific) have been identified [16, 21, 22]. One should have expected that theophylline-induced relaxation would be lower in presence of an impairment of cGMP relaxation. Since theophylline, in addition to its PDE inhibitory activity, is an antagonist at adenosine receptors, blockade of adenosine activity has been proposed as a possible mechanism for theophylline's effects. Accordingly, Ethier and Madison have shown that, in addition to its well-established indirect effects on smooth muscle cells, adenosine also has direct effects on contractile signalling pathways since it mobilizes intracellular calcium stores [23]. This may explain why theophylline has a very potent bronchodilating effects *ex vivo* using a high concentration.

#### *4.4. Clinical perspectives*

Establishing a formal relationship between pulmonary vascular and bronchial functions is an important issue. We further suggest that NO-related dysfunction may participate in this link. Whether pulmonary endothelial dysfunction is associated with COPD morbidity warrants further studies. But we recently evidenced a frequent endothelial dysfunction of *ex vivo* pulmonary arteries of patients suffering from cystic fibrosis (that was associated with pulmonary hypertension), and we further demonstrated that criteria of mild pulmonary

vascular disease were evidenced in some patients with cystic fibrosis that participated in exercise limitation [24].

Finally, it will deserve assessing the effects of statins on bronchial functions because reductions in COPD morbidity and mortality, and improvements in lung function, have been attributed to statin use. Insights into cellular mechanisms indicate that statins promote vasorelaxation partly by upregulating the expression of endothelial NO synthase [25].

#### *4.5. Conclusion*

This is the first study suggesting that endothelial dysfunction of pulmonary arteries is associated with increased resting tone and impaired cGMP-mediated dilation of airways in former smokers, suggesting common underlying mechanisms of pulmonary arterial and bronchial dysfunctions.

## **Acknowledgments**

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## **Conflict of Interest**

The authors declare no conflict of interest.

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## Figure legends

**Figure 1:** Acetylcholine-response of precontracted arterial rings.

Patients were divided according to their response to acetylcholine: endothelial dysfunction (grey circles) is defined by a constrictor response (negative values), while the normal response (white circle) is defined by dilation (positive values). Errors bars are 95% confidence interval. # denotes a *P* value < 0.05, \* denotes a *P* value < 0.005.

**Figure 2:** Relationship between acetylcholine-induced arterial and zaprinast-induced bronchial dilations.

Acetylcholine-induced dilation of arterial rings is expressed as percentage of the constrictor response to phenylephrine (PE), whereas zaprinast-induced dilation of bronchial rings is expressed as percentage of maximal response to theophylline (theo). An equivalent molar concentration ( $10^{-5}$  M) for both drugs is selected for the figure, but the relationship between zaprinast ( $10^{-5}$  M) response and ACh was of borderline significance for ACh  $10^{-7}$  M ( $p=0.057$ ) and significant for ACh  $10^{-6}$  M to  $10^{-4}$  M ( $p=0.022$ , figure and  $p=0.024$ , respectively). Grey circles are patients with endothelial dysfunction (negative acetylcholine-induced dilation = constriction,  $n=5$ ), whereas white circles are patients without endothelial dysfunction ( $n=15$ ).

**Figure 3:** Zaprinast-induced bronchodilation.

Patients are divided according the presence of endothelial dysfunction (grey bars, n=5) or its absence (white bars, n=15). Box and whisker plots show median, 25<sup>th</sup> and 75<sup>th</sup> percentile and 10<sup>th</sup> and 90<sup>th</sup> percentile.

Upper panel: Levels of tension (g) according to the condition, initial resting tone (RT1), constrictor response to acetylcholine ( $10^{-3}$  M Ach), resting tone after contraction (RT2), dose response to zaprinast ( $10^{-8}$  to  $10^{-5}$  M), and maximal dilation induced by theophylline ( $10^{-3}$  M theo). # denotes a *P* value < 0.05, \* denotes a *P* value < 0.01.

Patients without endothelial dysfunction depicted a weak but highly significant dilator response to zaprinast as compared RT-2 level:  $10^{-8}$  M zaprinast, *p*=0.009;  $10^{-7}$  M, *p*=0.020;  $10^{-6}$  M, *p*=0.011;  $10^{-5}$  M, *p*=0.002 (Wilcoxon paired test), whereas patients with dysfunction had no significant dilator response to zaprinast (*p*>0.05 for all concentrations, Wilcoxon test).

Lower panel: Response to zaprinast expressed as percentage of maximal theo-induced dilation ( $10^{-8}$  to  $10^{-5}$  M). A significant dilator response is evidenced in patients without endothelial dysfunction (trend for  $10^{-7}$ M, significant for  $10^{-5}$  M).

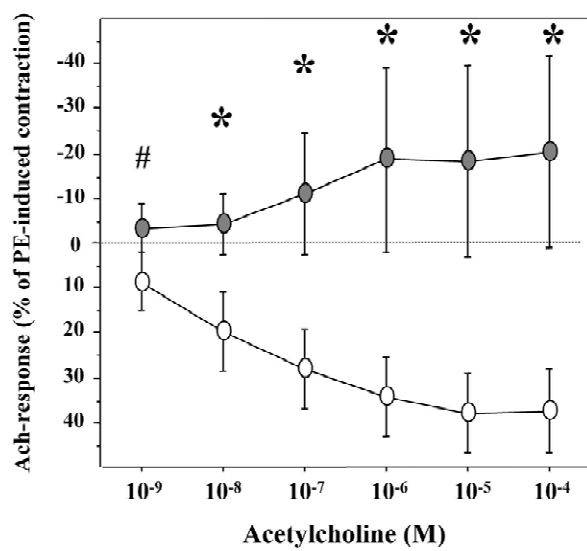
**Table 1.** Characteristics of the patients.

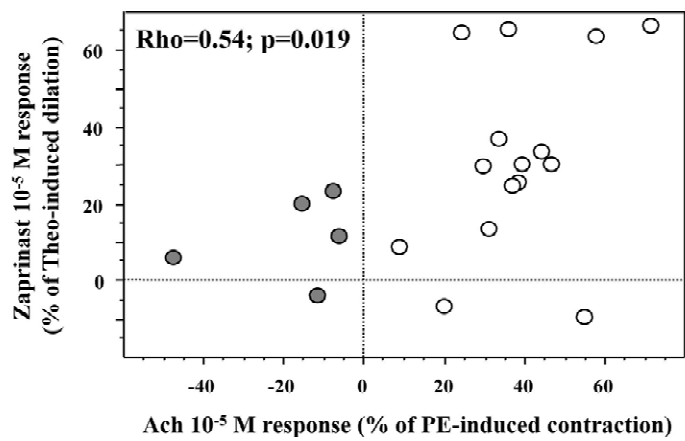
Characteristic	Patients with ED n = 5	Patients without ED n = 15	<i>P</i> value
Age, years	61 [51-66]	64 [58-70]	NS
Sex ratio, Female/Male	1/4	2/13	NS
Tobacco, Pack-years	30 [26-60]	50 [21-65]	NS
FEV <sub>1</sub> , % predicted	78 [72-99]	88 [75-99]	NS
Smokers without COPD, n	3	10	NS
GOLD class, n			NS
Class I	0	2	
Class II	2	3	
Preoperative chemotherapy, n	1	3	NS

Results are expressed as median [interquartile range]

ED denotes endothelial dysfunction; NS denotes non significant

FEV<sub>1</sub> denotes forced expiratory volume in one second (post-bronchodilator value)





ACCEPTED MANUSCRIPT

