

## Impairment of Pulmonary Vasoreactivity in Response to Endothelin-1 In Patients With Chronic Obstructive Pulmonary Disease (COPD)

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### Abstract

**Background:** Pulmonary hypertension secondary to COPD is characterized principally by impairment of vasoreactivity and vascular remodeling. While the structural changes have been described in all stages of COPD, the impairment of vasoreactivity in response to ET-1, a potent vasoconstrictor, has not been well understood.

**Materials and methods:** Proximal pulmonary arteries were obtained from non-smokers, smokers with normal lung function, and COPD patients who underwent lung resection for other diseases (n=6 in each group). Pulmonary arterial contraction induced by ET-1 was assessed without or with the presence of ET-1 receptor antagonists (BQ-123 and BQ-788). Expressions of ET-1 receptors were measured by immunohistochemistry, western blot, and qRT-PCR.

**Results:** ET-1-induced pulmonary arterial contraction increased significantly in COPD patients in comparison with control subjects and non-COPD smokers ( $P < 0.05$ ) and the use of ET-1 receptor antagonists could not prevent this contraction. The hypercontraction was not associated with the increase of ET-1 and ET-1 receptors expression in pulmonary arterial vessels.

**Conclusion:** In patients with COPD, pulmonary arterial contraction induced by ET-1 has increased significantly and could not be prevented by using ET-1 receptor antagonist alone. This alteration might be related to the upregulation of other signalling pathways in pulmonary arterial vessels of COPD patients.

**Keywords:** COPD; ET-1; ET-1 receptor antagonists; BQ-123; BQ-788

### Introduction

In patients with chronic obstructive pulmonary disease (COPD), the impairment of vasoreactivity in response to endothelium-derived constricting factor (endothelin-1, thromboxane) and relaxing factors (nitric oxide, prostacyclin) has not been well understood. This impairment might be due to endothelial dysfunction and might promote to the alteration of pulmonary vascular tone and pulmonary vascular hypertrophy in these patients [1]. Among these vasoreactivity factors, endothelin-1 (ET-1), the most potent endogenous vasoconstrictor peptide of endothelin family, produced by endothelial cells, has an important role in the modulation of vascular resistance.

Moreover, the abnormalities in endothelin-1 (ET-1) metabolism have been also reported in COPD patients. Previous studies showed that the concentration of ET-1 in plasma, in urinary excretion, and in saliva has increased significantly in COPD patients [2]. Further, at local level, the increase of endothelin expression has been founded in pulmonary vessels in patients with pulmonary hypertension secondary to COPD [3]. Although the effect of cigarette smoke on systemic vascular tone due to ET-1 by increasing the vasoconstriction has been well studied [4], the vasoreactivity of pulmonary vessels in response to ET-1 in healthy smokers and especially in smokers with COPD has not been well understood.

Generally, ET-1 exerts its vasoactive properties by binding to its specific receptor ET<sub>A</sub> presenting in pulmonary arterial smooth muscle cells (PASCs), and ET<sub>B</sub> expressing in endothelial cells (ECs) and in PASCs. Under normal conditions, ET-1 mediates Ca<sup>2+</sup> releasing

from sarcoplasmic reticulum, (PKC-dependent inhibition of voltage-gated K<sup>+</sup> (K<sub>v</sub>) channels), and subsequent membrane depolarization and Ca<sup>2+</sup> influx through voltage-dependent calcium channels [5-7]. This increase in intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) activates myosin light chain kinase (MLCK), leading to phosphorylation of myosin light chains and vasoconstriction [8, 9]. In chronic hypoxia exposure, where the ET-1 sensitivity and its receptor ET-A have been upregulated [10-12], ET-1-induced vasoconstriction is independently of intracellular calcium concentration and related mostly to Ca<sup>2+</sup>-sensitization [13]. Therefore, Ca<sup>2+</sup>-sensitization regulates vascular tone by increasing phosphorylation of myosin regulatory light chain (MLC<sub>20</sub>) [14]. The use of ET-1 receptor antagonists may prevent the vasoconstriction in hypoxia-induced pulmonary hypertension.

The aims of this study were to evaluate the vasoreactivity of pulmonary arteries in smokers with and without COPD in response to exogenous ET-1 and its receptor inhibitors.

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## Methods

### Subjects and tissue preparations

Subjects, who underwent lung resection for other diseases, enrolled in the study. Three groups of subjects were constituted: non-smokers (control subjects), healthy smokers (non-COPD smokers) and smokers with COPD (COPD patients). COPD were defined and classified by the international standard criteria [ratio of FEV<sub>1</sub> (forced-expiratory volume in one second)/FVC (forced vital capacity) <0.7] [15]. The study was approved by French Ethnical Committee and taken place in UPRES25511 – Paris Descartes University. All patients were informed about the aims of study and written informed consent was obtained from each subjects.

Proximal pulmonary arterial vessels were dissected precisely far away from local damaged tissues at operation room. Pulmonary arterial rings of 4 to 6 mm external diameter and 3 mm of length, cleaned of perivascular tissue, were dissected and immediately putted in Krebs-Henseleit solution at 4°C for vasoreactivity study within less than 30 minutes. The others dissected pulmonary arterial vessels were conserved at -80°C for proteins and RNA extractions or fixed in 4% formalin and embedded in paraffin for immunohistochemistry study.

### Chemical reagents and antibodies

All chemical reagents used in the present study were purchased from Sigma-Aldrich (Sigma-Aldrich France; St Quentin En Fallavier, France) unless otherwise noted. Primary antibody anti-ET<sub>A</sub> and ET<sub>B</sub> were purchased from Assay Designs Inc (Ann Arbor, USA).

For all washing steps in immunohistochemical staining and Western blot, phosphate buffered saline (PBS, pH 7.4) was used with Tween 20 (0.05%), noted as PBS-T. Primary antibodies and HRP-conjugated secondary antibodies for Western blot were purchased from Santa Cruz Biotechnology Incorporation (Santa Cruz, CA, USA), biotinylated secondary antibodies for immunohistochemical staining from Vector Laboratories (Burlingame, CA, USA), unless differently mentioned. Antibodies used in western blotting were diluted in non-fat milk (5%), Tween-20 (0.05%) in PBS and those for immunohistochemistry in PBS.

### Measurement of Pulmonary Vascular Contraction

Pulmonary arterial ring was suspended horizontally between two parallel stainless steel wires for the measurement of isometric tension by organ chambers (Emka Technologies, Paris, France). Pulmonary arterial ring was dipped in a fresh Krebs-Henseleit solution with the following composition: glucose 10 mM, pyruvate 2 mM, HEPES 10 mM, EDTA 0.03mM, NaCl 118 mM, KCl 4.7 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> 15 mM, and the solution was continuously oxygenated with 95% O<sub>2</sub>-5%CO<sub>2</sub> (Air Liquide Santé; Paris, France) and maintained at 37°C.

The variations of force generated by the ring segment were measured and recorded by IOX-Software (Emka Technologies, Paris, France). Pulmonary arterial rings were stabilized under controlled condition at 1.0 g of resting tension (for 10 min before stretching progressively at 1.5 g to obtain the pre-tension values as determined by experimental assessments), and it was corresponded to optimal resting tension for isometric condition registry. After the initial equilibration period, the vessels were exposed to a maximally effective concentration of 80 mM KCl. When a stable tension plateau developed, the KCl was removed by several washes and a period of 30 min was allowed

for reequilibration. Then, pulmonary arterial rings were exposed to increasing and cumulative doses of ET-1 (10<sup>-10</sup> to 10<sup>-7</sup>M), in the present of inhibitors of ET<sub>A</sub> receptor (BQ-123 at 10<sup>-6</sup>M) or ET<sub>B</sub> receptor (BQ-788 at 10<sup>-6</sup>M). Other pulmonary arterial rings of the same patients were incubated with receptor inhibitors during at least 1 hour before starting the measurement of ET-1 dose-response curve. The isometric contractions were normalized by expressing force developed per cross-sectional area (g/mm<sup>2</sup>), which took into account the variation in vascular sizes.

The integrity of pulmonary arterial endothelial cells was tested by the vasodilatation endothelium-dependent in response to 10<sup>-6</sup>M acetylcholine (Ach) at the end of ET-1 stimulation protocol and when the plateau of contraction curve had been obtained at 10<sup>-7</sup>M. The relaxation of more than 30% compared to the value before acetylcholine response was necessary to confirm the endothelial intact. The ET-1 response-curve had been presented as the percentage of maximal contraction induced by 80mM potassium chloride (KCl). For each patient, four pulmonary arterial rings were studied.

### Immunohistochemistry for ET<sub>A</sub> and ET<sub>B</sub> receptors

For immunohistochemical staining of ET<sub>A</sub> and ET<sub>B</sub> receptors, after re-hydration by successive baths of decreasing ethanol concentrations, all pulmonary arterial sections had been submerged in citrate buffer (10 mM citrate, 0.05% Tween, and pH 6.0) and unmasked with heat treatment in micro-oven (750W × 3 times). Endogenous peroxidase activity was neutralized by incubating with oxygenate water of 3% during 15 minutes. The non-specific endogenous binding sites had been blocked by 2% BSA (bovine serum albumin) in 45 minutes. After washing steps, pulmonary arterial sections had been incubated for one hour with primary antibodies, which had been diluted to 1/50 for anti-ET<sub>A</sub> and 1/25 for anti-ET<sub>B</sub> in PBS-T of 0.05% and 1% BSA as titration essays. Negative-control sections were incubated in blocking buffer alone without primary antibody. The sections had been then incubated with biotinylated secondary antibody at 1/200 dilution within 45 minutes. After successive washing steps, pulmonary arterial sections were incubated with preformed avidin-peroxydase RTU Vectastain Kit (Vector Laboratory; Burlingame, USA) before revealed with metal enhanced DAB peroxidase substrate (3, 3'-diaminobenzidine) until the desired staining was obtained (4 minutes for ET<sub>A</sub> and 5 minutes for ET<sub>B</sub>). Tissue sections were then counterstained with hematoxylin-Gill solution and examined using light microscopy. ET<sub>A</sub> and ET<sub>B</sub> immunostaining expression was measured in endothelial and SMC cells of proximal pulmonary arteries. The staining expression was measured by using intensity score. At least ten counted images were performed for each patient.

### Real time PCR for mRNA of ET-1, ET-1 conversion enzyme, and ET<sub>A</sub> and ET<sub>B</sub> receptors

Pulmonary arteries for real time PCR experiments were frozen in liquid nitrogen and stored at -80°C. The samples were homogenated and realized ARN extraction in RNase-free condition by using combined phenol-guanidine isothiocyanate buffer (Trizol<sup>®</sup>, Life technologies, CA, USA). Each sample had been homogenated in Trizol at 4°C with Ultraturax (T25, Staufen, Germany), centrifuged at 12000 × g within 10 minutes to eliminate tissue debris, and then added with chloroform (Merck, Darmstadt, Germany) to the lysate. After precipitated with isopropanol (Merck, Darmstadt, Germany) and centrifugated at 14000 × g (10 min), RNA pellet had been washed with 70% ethanol (Merck,

Hohenbrunn, Germany) and centrifugated at 12000 × g. Pellet had been then redissolve in RNase-free water and quantified with optical density at 260 nm.

After RNA isolation, reverse transcription was carried out in mixed solution of 7.5 μM hexanucleotide, 9 mM dithiothreitol, 20 U RNase inhibitor (Perkin-Elmer), 4 dNTP with the concentration of 220 μM for each (Boehringer), and 50 U Reverse Transcriptase (Superscript; Gibco BRL). It had been placed in PCR apparatus (Perkin-Elmer Gene Amp PCR system 2400, CA, USA) with following cycles: 10 min at 22°C, 1 hour at 42°C, and 5 min at 99°C. Real time PCR was performed with the real time PCR GeneAmp machine 7700. The GeneAmp machine 7700 sequence detection system monitored the binding of a fluorescent dye to double-strand DNA by real time detection of the fluorescence during each cycle of PCR amplification. Control samples without the reverse-transcription step and no added RNA were also included in each plate to detect any possible contamination. The housekeeping genes, HPRT (Hypoxanthine PhosphoRibosyl Transférase) was used as references. The real time PCR reaction was performed at a temperature of 50°C for 2 min, 95°C for 10 min, and the following 40 PCR cycles with 95°C for 15 s and 60°C for 1 min, and fluorescence was measured after amplification step.

The sequences of the primer pairs were designed as follows: ET-1: forward 5'-CTTCTGCCACCTGGACATCA- 3' and reverse 5'-GGCTTCCAAGTCCATACGGA-3'; ET<sub>A</sub> receptor: forward 5'-TG-GTGTGCACTGCGATCTTC-3' and reverse 5'-GCAATTCT-CAAGCTGCCATTC-3'; ET<sub>B</sub> receptor: forward 5'-TGGCG-TATTTCTTTGCAAGCT-3' and reverse 5'-AGGTTGAGGACGGT-GATCCC-3'; ECE-1: forward 5'-TGGGGGACCTTCAGCAACCT-3', reverse 5'-GGGTGCTGGAGTTGCTCTTG-3'; HPRT: forward 5'-TCAGGCAGTATAATCAAAGATGGT-3' and reverse 5'-AGTCTGGCTTATATCCAACACTTCG-3'.

At the end of each PCR run, the data were automatically analyzed by the system and an amplification plot was generated for each DNA sample. Quantitative real-time PCR values are expressed as the fold change of the target-gene expression relative to HPRT mRNA in each sample using the following formula: fold change = 2<sup>-ΔΔC<sub>T</sub></sup>, where ΔC<sub>T</sub> = C<sub>Target gene</sub> - C<sub>HPRT</sub> and -ΔΔC<sub>T</sub> = ΔC<sub>Test sample</sub> - ΔC<sub>Tcontrol</sub>. The experimental approach was further validated by the observation that the differences between the C<sub>T</sub> for the target gene and β-actin remained essentially constant for each starting DNA amount.

### Western blot analysis for ET-1 receptors (ET<sub>A</sub>, ET<sub>B</sub>)

Frozen pulmonary arterial samples were homogenized in ice-cold RIPA buffer (Cell Signalling Technology, Danvers, USA). The homogenates were centrifuged at 21000 g at 4°C for 20 minutes to remove tissue debris. Protein contents of homogenated lysates were measured by BCA Protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of lysates (30 μg of total protein content per sample), were subjected to electrophoresis on SDS-PAGE gels (12% for ET<sub>A</sub> and ET<sub>B</sub>), and then transferred to PVDF membranes (Immobilon-P, Millipore, MA, USA). Membranes were blocked in 5% non-fat milk, 0.1% Twen-20 in PBS for 1 hour at room temperature and then hybridized overnight at 4°C with primary antibodies diluted at 1/500. After being washed in PBS-T, membranes were incubated with HRP-linked goat anti-rabbit, diluted at 1/10000. Protein bands were developed on the film by using Enhanced ChemiLuminescence Plus reagent, according to the manufacturer's instructions (Amersham, Biosciences, Orsay, France). The protein density was quantified by using Image Software System (Genius 2). The equal amounts of proteins

loaded per well were verified by stripping the blots with Tris-HCl (pH 6.7) 62.5 mM, SDS 2%, and β-mercapto-ethanol 100mM for 30 minutes at 55°C. The membranes were reprobed with a mouse polyclonal anti-β-actin antibody (1/1000; sc-9104, Santa Cruz Biotechnology) and then incubated with HRP-linked goat anti-rabbit, diluted at 1/10000. Results were expressed as ratios of ET<sub>A</sub> and ET<sub>B</sub>/β-actin.

### Statistical analysis

Statistical comparisons were performed with non parametric Kruskal-Wallis test and with analysis of variance (ANOVA) among more than three groups by using the SPSS 16.0 software (Chicago, IL). Data were presented as means ± SD. Differences were considered significant with P < 0.05.

## Results

### Clinical characteristics

Three groups of patients who met the inclusion criteria were included in the present study (6 subjects for each group). The clinical and functional characteristics of the patients are presented in Table 1. The tobacco consumption in COPD patients was higher than non-COPD smokers (P<0.05). The non-COPD smokers had a normal lung function with FEV<sub>1</sub> = 97 ± 5% and FEV<sub>1</sub>/FVC=75 ± 4%. COPD patients had moderate airflow obstruction with FEV<sub>1</sub>=73±15% and FEV<sub>1</sub>/FVC=64±6%. All the patients of three groups had a normal gas blood exchange.

### Vasoconstriction induced by ET-1

The results of contraction induced by ET-1 with increasing concentration (10<sup>-10</sup>-10<sup>-7</sup> M) in organ bath chambers showed that in compare to Emax (contraction induced by 80 mM KCl) and with the high dose (10<sup>-8</sup>-10<sup>-7</sup> M), the percentage of maximal contraction was significantly higher in COPD patients than controls and non-COPD smokers (P<0.05; Figure 1). Whereas, with the lose dose (10<sup>-10</sup>-10<sup>-9</sup> M), there were not any significant differences between three groups in response to ET-1 (P>0.05; Figure 1).

### Effect of ET<sub>A</sub> and ET<sub>B</sub> antagonists in ET-1-induced contraction

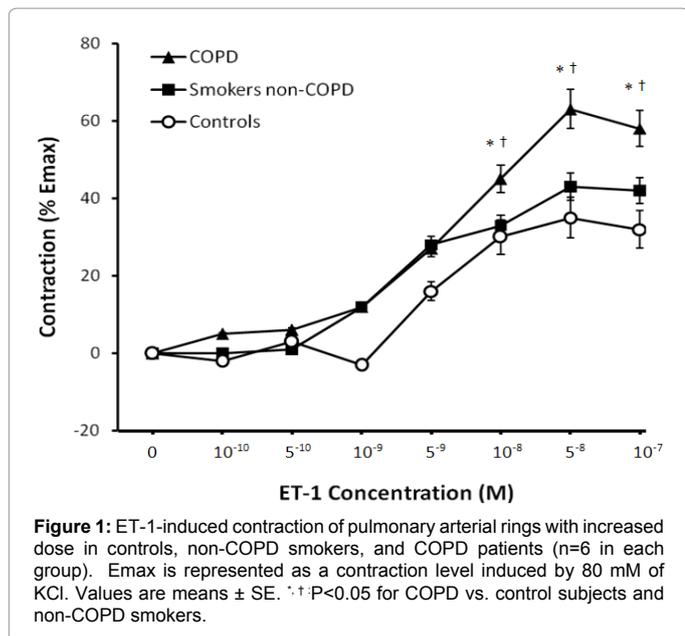
To determine the role of ET<sub>A</sub> or ET<sub>B</sub> receptor involved in the ET-1 induced-contraction, in the present study, the pulmonary arterial rings had been exposed to selective antagonist of ET<sub>A</sub> receptor (BQ-123 at 10<sup>-6</sup>M) and ET<sub>B</sub> receptor (BQ-788 at 10<sup>-6</sup>M) in organ bath chambers.

In control group, in the presence of BQ-123 and BQ-788, the percentage of pulmonary arterial contraction induced by ET-1 was

Variables	Control (non smoking) (n = 6)	Smokers non-COPD (n = 6)	COPD (n = 6)	P
Age, years	59 ± 7	50 ± 10	67 ± 12	NS <sup>†‡</sup>
Male/Female	2/4	3/3	3/3	
Tobacco, pack-year	-	30 ± 13	41 ± 23	< 0.05 <sup>‡</sup>
FEV <sub>1</sub> , % pred	97 ± 5	97 ± 5	73 ± 15	< 0.05 <sup>†‡</sup>
FEV <sub>1</sub> /FVC	86 ± 5	75 ± 4	64 ± 6	NS, < 0.05 <sup>†‡</sup>
FVC, % pred	105 ± 17	104 ± 9	87 ± 22	NS <sup>†‡</sup>
PaO <sub>2</sub> , mmHg	86 ± 11	87 ± 17	83 ± 14	NS <sup>†‡</sup>
PaCO <sub>2</sub> , mmHg	38 ± 2	37 ± 2	37 ± 5	NS <sup>†‡</sup>

(%pred : percentage of predicted value; FEV<sub>1</sub> indicates forced expiratory volume in one second; FVC, forced vital capacity; \*: non-COPD smokers vs controls; †: COPD vs control subjects; ‡: COPD vs non-COPD smokers; NS: no significant)

Table 1: Clinical characteristics of study subjects.



significantly reduced in comparison without antagonists ( $P<0.05$ ; Figure 2a). In non-COPD smokers, the contraction had been reduced significantly in the presence of BQ-123. Whereas, in the presence of BQ-788, the pulmonary arterial contraction induced by ET-1 had two phases: reduced the contraction at low dose ( $10^{-10}$ - $10^{-8}$ M), and increased the contraction at high dose ( $10^{-8}$ - $10^{-7}$ M); but there were not any significant differences in comparison with ET-1-induced contraction without BQ-788 ( $P>0.05$ ; Figure 2b). In COPD patients, the reductions of pulmonary arterial contraction induced by ET-1 in the presence of BQ-123 and BQ-788 were not significantly different in comparison with ET-1-induced contraction without antagonists ( $P>0.05$ ; Figure 2c).

### Semi-quantitative and quantitative assessment of ET<sub>A</sub> and ET<sub>B</sub> receptors

**Immunohistochemistry:** The results of immunostaining ET<sub>A</sub> and ET<sub>B</sub> receptor expression in pulmonary arterial sections showed that there were no significant differences between non-COPD and COPD smokers for expression intensity per surface unit assessment and in comparison with control group ( $P>0.05$ ; Figure 3a).

**Western blot:** The protein expression of ET<sub>A</sub> and ET<sub>B</sub> receptor measured by densitometry showed that there were no significant differences between three groups for protein levels ( $P>0.05$ ; Figure 3b).

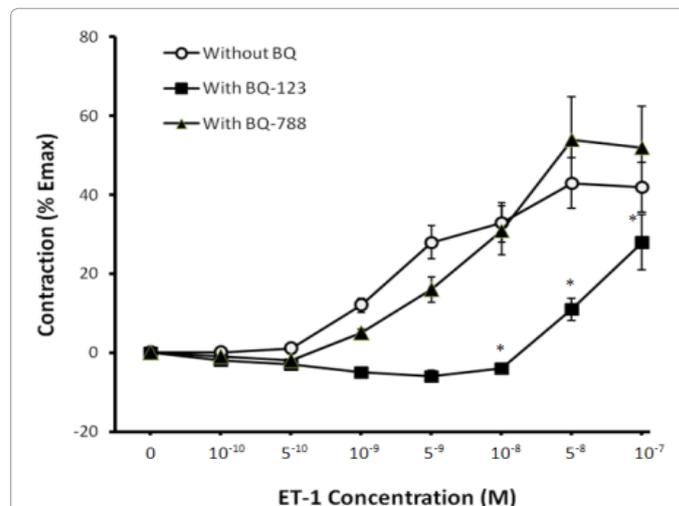
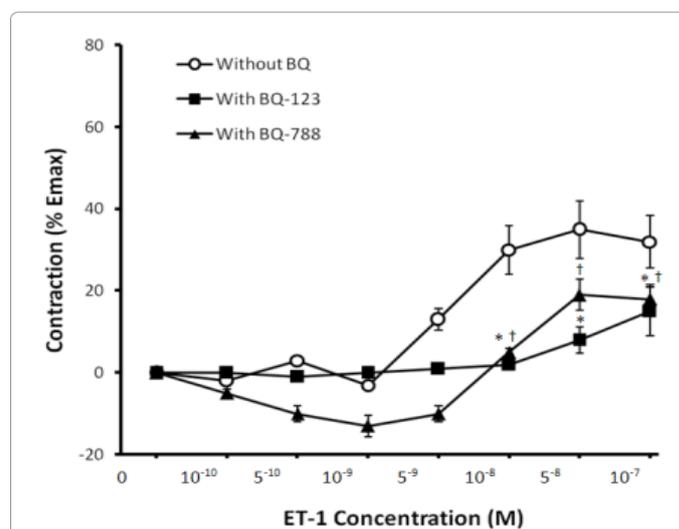
**Real time quantity-PCR (RTq-PCR):** Although the contractions of pulmonary arterial rings induced by ET-1 were different between non-COPD and COPD smokers, the results of RTq-PCR showed that there were no significant differences between three groups for the transcription of mRNA ( $P>0.05$ ; Figure 3c).

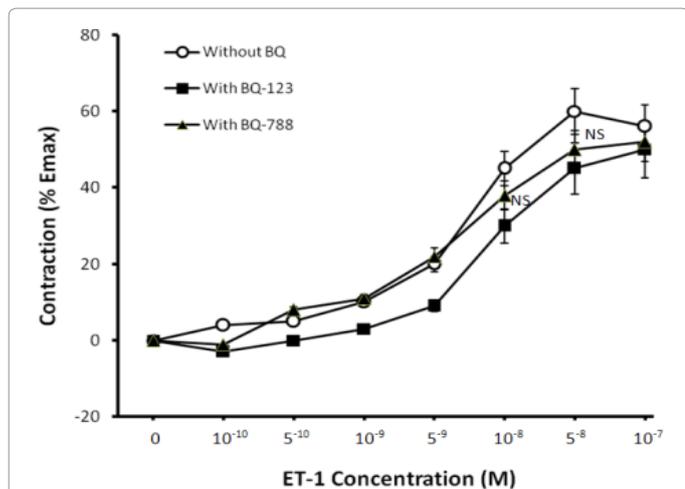
The relative expressions of ET-1 and ET-1 enzyme conversion (EEC) were not significant different between three groups. Although the mRNA expression of ET-1 and EEC in non-COPD smokers were slightly higher than controls and COPD patients, but there were not any significant differences ( $P>0.05$ ; data not shown).

### Discussion

The aim of this study was to elucidate the role of ET<sub>A</sub> and ET<sub>B</sub> receptors in pulmonary arteries responding to ET-1 induced vasoconstriction in non-COPD smokers and in COPD patients. The results of present study showed that: (1) in COPD patients, the pulmonary vasoconstriction induced by ET-1 was significantly increased in comparison with non-smokers and non-COPD smokers; (2) ET-1-induced vasoconstriction was mediated by ET<sub>A</sub> and ET<sub>B</sub> in non-smokers and predominantly by ET<sub>A</sub> in non-COPD smokers; (3) in COPD patients, vasoconstriction induced by ET-1 was not completely inhibited by selective ET-1 receptor antagonists.

Until now, the effect of cigarette smoking on the tonus of

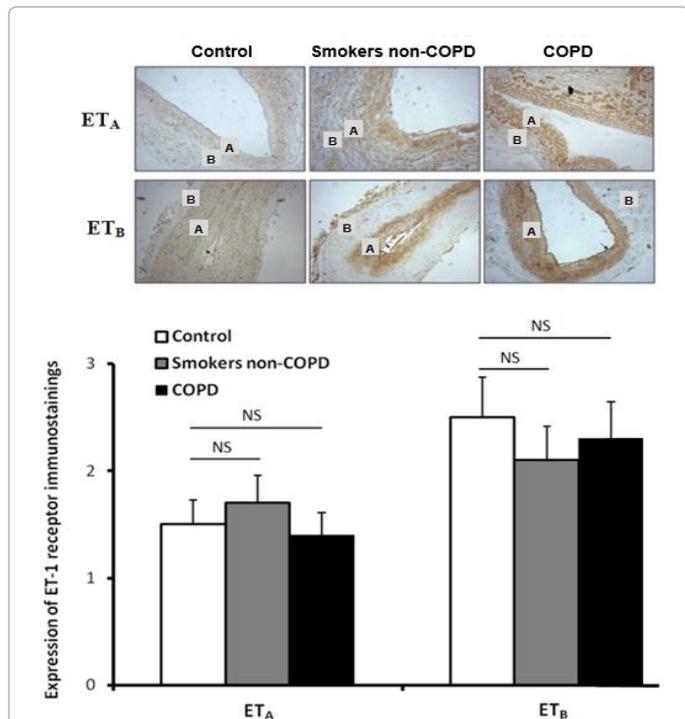




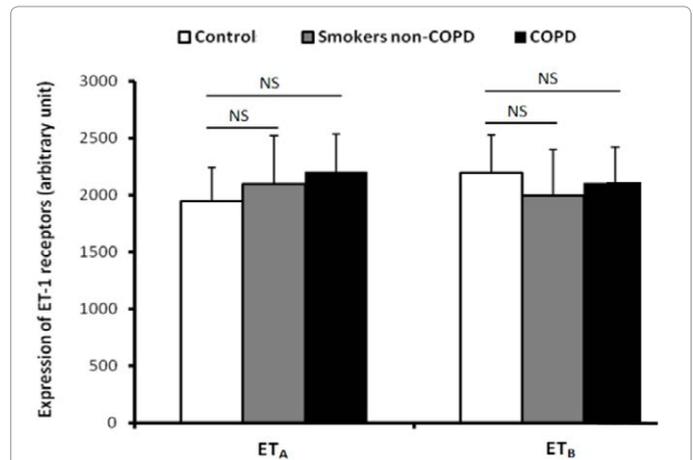
**Figure 2c:** ET-1-induced contraction of pulmonary arterial rings with increased dose in the presence of ET<sub>A</sub> (BQ-123) and ET<sub>B</sub> (BQ-788) antagonists in COPD patients (4 measurement for each patient). Emax is represented as a contraction level induced by 80 mM of KCl. Values are means ± SE. NS: no significant differences with P>0.05 for BQ-123 and BQ-788 vs without antagonists.

the endothelium-derived contracting factors, ET-1 has an important role. The previous studies showed that the plasma concentration of ET-1 and the expression of ET<sub>A</sub> receptor have been increased in chronic hypoxic exposure and in COPD with hypoxia, and it was associated with the alteration of ET-1-induced vasoconstriction [17-19]. The mechanism by which ET-1 is upregulated in COPD has not been completely understood. It might be partially mediated by oxidative stress due to cigarette smoke or by alveolar hypoxia when the disease is in progress. In the present study, although ET-1-induced pulmonary vasoconstriction had been found in all the three groups, the vasoconstriction in response to ET-1 at high doses ( $\geq 10^{-8}$  M) was significantly higher in COPD patients than control subjects and non-COPD smokers (Figure 1). Whereas, ET-1-induced vasoconstriction in non-COPD smokers was slightly higher than control, but there were no significant difference (Figure 1). These results suggested that the contractility of pulmonary arteries with ET-1 was higher in COPD patients.

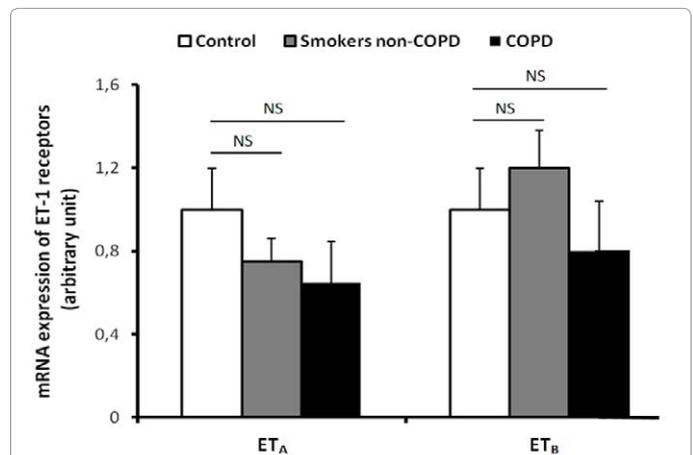
In the present study, the use of ET-1 antagonists reduced significantly the contraction induced by ET-1 in non-smokers (BQ-123 for ET<sub>A</sub> and



**Figure 3a:** Expression of ET<sub>A</sub> and ET<sub>B</sub> receptors measured by immunohistochemistry in pulmonary arterial vessels in control, non-COPD smokers, and COPD patients (magnification  $\times 10$ ). There were no significant differences between three groups (n=6 in each group). A: adventi; B: media. NS: no significant difference vs. control subjects with P>0.05.



**Figure 3b:** Expression of ET<sub>A</sub> and ET<sub>B</sub> receptors measured by western blot in pulmonary arterial vessels in control, non-COPD smokers, and COPD patients. There were no significant differences between three groups (n=6 in each group). NS: no significant difference vs. control subjects with P>0.05.



**Figure 3c:** Expression of ET<sub>A</sub> and ET<sub>B</sub> mRNA quantified by RT-PCR in pulmonary arterial vessels in control, non-COPD smokers, and COPD patients. There were no significant differences between three groups (n=6 in each group). NS: no significant difference vs. control subjects with P>0.05.

pulmonary arterial vessels induced by ET-1 in COPD has not been completely clarified. In COPD, the pulmonary vascular tone has been changed progressively due to the disturbance of vasodilatation – vasoconstriction balance. This event starts at early stage of disease and becomes more severe in advanced stage patients where the alveolar hypoxia and vascular remodelling has been established [16]. Among

BQ-788 for ET<sub>B</sub>) and in non-COPD smokers (BQ-123 for ET<sub>A</sub>) (Figure 2a-b). But the contractions were not different in COPD patients (Figure 2c). It confirmed that in non-smokers, the vasoconstriction responded to ET-1 was completely mediated by two receptor types (ET<sub>A</sub> and ET<sub>B</sub>) and the selective antagonists abolished this effect. However, in non-COPD smokers, ET<sub>A</sub> receptor had a predominant role in ET-1-induced pulmonary vasoconstriction (Figure 2b). Particularly, in COPD patients, although the contraction induced by ET-1 at a high dose was significantly higher than non-smokers and non-COPD smokers, ET-1 receptor antagonists could not reduce significantly the contraction (Figure 2c). In addition, in the present study, the mRNA expression of ET-1 and its receptors ET<sub>A</sub> and ET<sub>B</sub> were not different between three groups (Figure 3a-c). It suggested that in COPD, the pulmonary arterial contraction in response to ET-1 was not related directly to the upregulation of ET<sub>A</sub> and ET<sub>B</sub> receptors. In our study, we could not measure the plasma concentration of ET-1 of study patients to assess the correlation between the level of mRNA-ET-1 expression in pulmonary arterial vessels and the plasma concentration of ET-1. As discussed above, the plasma concentration of ET-1 was increased and associated with the upregulation of ET<sub>A</sub> expression and the alteration of vasoconstriction in COPD with hypoxemia. These data may explain why in our study, gene expression of ET-1 and its receptors was not increased significantly in COPD without hypoxemia.

In this study, the use of selective ET-1 receptor antagonists did not decrease the contraction of pulmonary arterial vessels induced by ET-1 in non-COPD smokers (for BQ788) and in COPD patients (for both BQ123 and BQ789) suggested that another pathway had been implicated in this phenomena. Under normal condition, vasoconstriction induced by ET-1 is mediated by Ca<sup>2+</sup> influx through L-type Ca<sup>2+</sup> channels and Ca<sup>2+</sup> release from the sarcoplasmic reticulum [20]. In some conditions such as in hypoxia exposure, ET-1-induced contraction is independent of changes in [Ca<sup>2+</sup>]<sub>i</sub> [21], and known as Ca<sup>2+</sup> sensitization of the contractile apparatus.

In summary, the results of present study might be explained that in non-smokers (control subjects), ET-1-induced pulmonary arterial contraction was mediated by ET<sub>A</sub> and ET<sub>B</sub> receptors and this contraction was inhibited by the use of either ET<sub>A</sub> or ET<sub>B</sub> receptor antagonist. In non-COPD smokers, ET-1-induced pulmonary arterial contraction was mediated principally via ET<sub>A</sub> receptor. Whereas, in COPD patients, either ET<sub>A</sub> or ET<sub>B</sub> receptor antagonist could not decrease significantly the contraction induced by ET-1. In these patients, the ET-1-induced pulmonary arterial contraction was not mediated via ET<sub>A</sub> or ET<sub>B</sub> receptor. In these patients, when stimulated with ET-1, the vasoconstriction might be mediated by another signaling pathway such as RhoA/Rho-kinase pathway. The activity of this pathway has been upregulated in healthy smoker and smokers with COPD [22, 23] and the use of Rho-kinase inhibitor might attenuated significantly the effect of ET-1 [24].

## Conclusion

Although carried out in the limit of patient number, the present study demonstrated that in patients with COPD, the contraction of pulmonary arterial vessels induced by ET-1 was not completely mediated via ET-1 receptors. Especially, in these patients, the hypercontraction induced by ET-1 was not associated with the upregulation of the mRNA expression of ET-1. However, others studies must be done to clarify the role of other pathways in ET-1-induced pulmonary arterial vasoconstriction in patients with COPD.

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