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Bosentan inhibits cigarette smoke-induced ET receptor expression in pulmonary arteries

Javier Milara,[1,7,*] Elena Gabarda,[2,*] Gustavo Juan,[3,4] José L. Ortiz,[2] Ricardo Guijarro,[3, 5] Miguel Martorell,[6] Esteban J. Morcillo,[2,7,8] Julio Cortijo.[1,2,7]

[1]Clinic research unit (UIC), University General Hospital Consortium, Valencia, Spain

[2]Department of Pharmacology, Faculty of Medicine, University of Valencia, Spain

[3]Department of Medicine, Faculty of Medicine, University of Valencia, Spain

[4]Respiratory Unit, University General Hospital Consortium, Valencia, Spain

[5]Thoracic Surgery Unit, University General Hospital Consortium, Valencia, Spain

[6]Department of pathology, University General Hospital Consortium, Valencia, Spain

[7]CIBERES, Health Institute Carlos III, Valencia, Spain

[8]Clinical Pharmacology Unit, University Clinic Hospital, Valencia, Spain

*****These authors contributed equally to this work

Corresponding Author: Javier Milara, PhD., Unidad de Investigación, Consorcio Hospital General Universitario, Avenida tres cruces s/n, E-46014 Valencia, Spain. Phone: +34 620231549, Fax: +34961972145, E-mail: xmilara@hotmail.com

Running Head: Bosentan inhibits CSE-induced ETR expression

Abstract

Endothelin system contributes to lung vascular tension and remodeling in smokers and chronic obstructive pulmonary disease (COPD) patients. This study examines the effect of cigarette smoke on endothelin receptor A (ETA) and B (ETB) expression, in human pulmonary artery smooth muscle cells (HPASMCs) and human small intrapulmonary arteries as well as their functional consequences.

Cigarette smoke extract increased ETA and ETB expression in HPASMCs and small intrapulmonary arteries which were attenuated by bosentan, the ETA antagonist BQ123 the ETB antagonist BQ788 and by blocking ET-1 with a monoclonal antibody against ET-1, suggesting a feed-forward mechanism mediated by ET-1 release. Endothelin receptor antagonism attenuated the cigarette smoke extract-induced HPASMC proliferation. Furthermore, cigarette smoke extract exposure increased the acute ET-1-induced small intrapulmonary artery contraction which was attenuated by Bosentan, BQ123 and BQ788. Pulmonary arteries from smokers and COPD patients showed a higher expression of ETA and ETB than those of non-smoker patients. These results show a novel mechanism by which endothelin receptor blockade attenuates cigarette smoke-induced endothelin receptor over-expression and subsequently small intrapulmonary artery tension. These data may be of potential value to explain therapeutic effects of bosentan in some forms of disproportionate pulmonary hypertension in COPD patients.

Key words: Bosentan; cigarette smoke; Endothelin receptor; human pulmonary artery smooth muscle cells; precision lung cut slice.

Introduction

Pulmonary hypertension (PH) is a relatively common form of cigarette smoke (CS)-induced lung disease. PH develops in ~ 6% of subjects with chronic obstructive pulmonary disease (COPD) but is present in ~ 40% of patients with a forced expiratory volume of <1 liter in 1s [1, 2]. The pathogenesis of PH in COPD is unclear. Current studies suggest that PH is caused by the direct effects of CS on the intrapulmonary vessels by the secretion of a number of vasoconstrictive /proliferative peptides such as endothelin (ET) or vascular endothelial growth factor (VEGF) which subsequently contribute to vascular remodeling and the development of PH [3]. The circulating levels of ET-1 are elevated after exposure to CS in humans [4], and it has been shown that ET-1 correlates with pulmonary systolic pressure in COPD patients with PH [5] suggesting that ET-1 is involved in CS-induced vascular remodeling. ET-1 activates two receptors, endothelin type A (ETA) and endothelin type B (ETB) receptors which are located in pulmonary artery smooth muscle cells (PASMCs), whilst exclusively ETB are present in endothelial cells. Both, ETA and ETB mediate proliferation and contractility in PASMCs from small pulmonary arteries whilst endothelial ETB mediates vasodilation in normal pulmonary arteries. ETB up-regulation has been observed in human blood vessels from patients with ischemic heart disease [6], hypertension [7] and severe PH [8]. More recently it has been shown that CS extract (CSE) induces ETA and ETB over-expression in resistant cerebral arteries from rats by a mechanism implicating the activation of intracellular mitogen-activated protein kinase (MAPK) ERK1/2 and p38, the transcription factor nuclear factor-kappa B (NF-κB) and c-Jun N-terminal kinase (JNK) [9-11].

The data suggest that the up-regulation of ETR is an important molecular mechanism that could play an essential part in the development of pathologic lung arteries

secondary to CS such as intimal thickening, vessel narrowing and pulmonary hypertension. Therefore, therapeutic interventions focused on inhibiting ETR expression could be of potential value to ameliorate pulmonary artery remodeling and tension in smokers with COPD. Currently, no data exists concerning the effect of CS on ETR expression in HPASMCs and human small intrapulmonary arteries and on the effect of ETR antagonists on the regulation of ETR.

This study was conducted to analyze the effect of cigarette smoke on ETR expression and ET release in HPASMCs and small intrapulmonary arteries, as well as the consequences of ETR up-regulation on HPASMC proliferation and small intrapulmonary artery tension. Furthermore, we studied whether ETR antagonism may attenuate ETR expression induced by cigarette smoke and their functional consequences. We found that the dual ETR antagonist bosentan, partially suppress the cigarette smoke-induced ET system activation in HPASMC and small intrapulmonary arteries as well as their functional consequences. These data may be of potential value to explain therapeutic effects of bosentan in some forms of disproportionate pulmonary hypertension in smokers with COPD.

Methods

See online supplement for further details

Patients

A total of 6 non-smoker controls, 7 smokers and 8 COPD patients were included in the study. All lung tissues studied in this work were taken from uninvolved lung tissue during lobectomy resection for malignant lesions. Samples of distal lung, located as far away as possible from the tumor, were chosen for the study. Pulmonary function tests

(forced spirometry) and arterial blood gas measurements were performed during the days prior to surgery. None of the patients exhibited clinical evidence of pulmonary hypertension. HPASMCs for *in vitro* experiments were isolated from 2-3mm pulmonary arteries of non-smoker lung tissue. Precision lung cut slices with small intrapulmonary arteries (100-300μm of internal diameter) were prepared from non-smoker lung tissue. Isolated pulmonary arteries of 0.5-1mm of internal diameter were used to measure ETR expression in non-smokers, smokers and COPD patients. The protocol was approved by the local research and independent ethics committee of the University General Hospital of Valencia. Informed written consent was obtained from each participant. Clinical features of patients are defined in table 1.

Isolation and culture of human pulmonary artery smooth muscle cells

Tumour free material surgical specimens from non-smoker patients were used. HPASMC were isolated from surgical specimens of human pulmonary arteries as previously outlined [12]. Briefly, segments of pulmonary artery (2-3 mm internal diameter) were digested with 1% collagenase (Gibco, UK) in RPMI-1640 culture medium for 30 min at 37°C. HPASMCs were isolated from HPAEC by means of CD-31-coated Dynabeads (Dynal Biotech, Germany) as previously outlined [12] and cultured in DMEM supplemented with 10% foetal calf serum (FCS), 1% fungizone, and 2% streptomycin/penicillin. All HPASMCs studied in this work were used from passage 1 to passage 4.

Real time RT–PCR

Real time RT-PCR was performed as previously outlined [13]. In brief, total RNA was isolated from cultured HPASMCs by using TriPure® Isolation Reagent (Roche,

Indianapolis, USA). cDNA was amplified with specific primers for ETB and ETA (pre-designed by Applied Biosystems, ETB: cat. nº: Hs00240747_m1; ETA: cat. nº: Hs03988672_m1) and GAPDH (pre-designed by Applied Biosystems, cat. nº: 4352339E) as a housekeeping. Relative quantification of these different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using GAPDH as the endogenous control and normalized to control group.

Western blot

Protein extraction from HPASMCs, human lung precision cut slices and pulmonary artery tissues from non-smokers, smokers and COPD patients were tested for ETB (50 kD), ETA (54 kD), p-ERK1/2 (42-44 kD) and β-actin (42 kD) using immunodetection western blot as previously outlined [13, 14]. Details are given in the Supplementary material online, Expanded Methods.

Preparation of cigarette smoke extracts solutions

CSE was prepared as we previously reported [13]. Briefly, the smoke of a research cigarette (2R4F; KY, USA) was generated by a respiratory pump and bubbled into a flask containing 25 ml of DMEM medium. The resultant CSE solution was deemed to be 100% CSE and was used for experiments within 30 min of preparation.

To test for cytotoxicity from CSE, HPASMCs were treated with CSE concentrations of up to 10% for 24 and 48 h. No significant difference in the lactate dehydrogenase supernatant level (lactate dehydrogenase cytotoxicity assay; Cayman, Spain) was observed, compared with the control group (data not shown).

Intracellular free Ca^{2+} measurements

Intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured by fluorescence microscopy (Nikon TE200, Tokyo, Japan) in HPASMCs using the Ca^{2+} indicator dye fura-2 as previously outlined [13-15]. The fura-2 fluorescence ratio was recorded every 0.1 s using Lambda 10-2 Sutter Instrument (Nikon CO. Tokyo, Japan) and fluorescence analysis was performed with the software Metafluor® 5.0. $[Ca^{2+}]_i$ was calculated by ratiometric analysis as previously mentioned [13].

Cell contraction

Contraction of HPASMCs in response to ET-1 was studied by traction microscopy as previously outlined [13]. Collagen-coated polyacrilamide gels with embedded fluorescent microbeads (200-nm diameter) were used. Gel disks with cultured HPASMCs were incubated for 24 h in absence (control) or presence of CSE 10% alone or in combination (30 min before CSE) with bosentan (10μM), BQ788 (10μM) and BQ123 (10μM). Later, gel disks with cultured HPASMCs were visualised with microscope using bright-field illumination. After 7 min of baseline recording, ET-1 (10 nM) was added, and fluorescent images were acquired for an additional 12 min. Traction forces exerted by the cell on the substrate were computed from the displacement field of the gel substrate.

Cell proliferation assay

HPASMC proliferation was measured by colorimetric immunoassay based on BrdU incorporation during DNA synthesis using a cell proliferation enzyme-linked immunosorbent assay BrdU kit (Roche, Mannheim, Germany; Catalogue no. 11647229001) as previously outlined [14]. In brief, HPASMCs

Rho Activity and ET Assays

A commercially available, enzyme-linked immunosorbent assay (ELISA)-based RhoA-GTP activity assay (G-LISA; Cytoskeleton, Denver, CO) was used to measure the relative RhoA-GTP activity of serum-starved HPASMCs after experimental treatments as previously outlined [13]. ET was measured in HPASMC culture supernatants by enzyme immune assay kit (Cayman chemical, USA) according to the manufacturer's protocol.

DCFDA fluorescence measurement of reactive oxygen species

Intracellular ROS levels (H_2O_2 and superoxide anion) were measured in HPASMCs by means of DCFDA as previously outlined [13]. Cells were treated with Bosentan (10μM), BQ788 (10μM) or BQ123 (10μM) 30 min prior to the addition of CSE 10% and remained together for 24h.

At the end of the incubation period cells were washed twice with PBS and fluorescence was measured using a microplate spectrophotometer (Victor 1420, PerkinElmer). Results were expressed as DFC fluorescence in relative fluorescence units (RFU). Representative pictures of each condition were taken *via* fluorescence microscopy.

Immunofluorescence

Precision human lung cut slices were incubated in the presence or absence of bosentan, BQ788 or BQ123 for 1 h and stimulated with CSE 10% for 24 h. Then, lung slices were washed three times with PBS and fixed (4% paraformaldehyde, 4h, at room temperature). Slices were included in O.C.T™ compound (tissue-Tek, USA) and immunostained with ETA and ETB antibodies followed by the appliance of secondary rhodamine fluorescence antibody. A comparative study of the autofluorescence of the

internal elastic lamina and external elastic lamina enabled the distinction between endothelial and smooth muscle cells in small intrapulmonary arteries.

Preparation of precision-cut lung slices from resected human Lung

Precision lung cut slices were obtained as previously outlined with minor modifications [16]. Briefly, 3% (w/v) ultralow melting point agarose (Sigma, UK) was injected into the lung tissue which was cut into precision-cut slices using a Krumdieck tissue slicer (model no. MD4000; Alabama Research, AL) with the slice thickness set at 260 to 300 μm. We carefully chose only small arteries that were adjacent to identifiable small airways. Sections were placed in fresh media and incubated in the presence or absence of bosentan (10μM), BQ788 (10μM) or BQ123 (10μM) for 1h prior to stimulation with or without CSE 10% for 24h. The slice was visualised using a microscope (Nikon Eclipse TE200; magnification, x40) connected to a live CCD camera CoolSNAPfx photometrics. After washout (30 min), KCl 80mM was perfused during 5 min so as to establish the maximal contractile response (100%). After rinsing and equilibration (normally 10min of perfusion) the lowest concentration of ET-1 to begin the concentration response (10^{-9} to 10^{-6} M) was administered. Small pulmonary artery contraction was continuously monitored and was expressed as a percentage of the maximal reduction of area obtained with KCl. Artery lumen area was measured using MetaMorph software (Molecular Devices, USA) and given in units of square micrometers. Results were expressed as % of KCl area. A log EC_{50} value and maximum drug effect (Emax) value for each artery was derived from a concentration-response curve. Details are given in the Supplementary material online, Expanded Methods.

Analysis of results

All values are reported as mean+SEM. Determinations were performed in duplicate and at least three independent experiments were performed for each set of conditions. Two-group comparisons were analysed using the two-tailed Student's paired t-test for dependent samples, or unpaired t-test for independent samples. Multiple comparisons were analysed by one-way analysis of variance followed by Student–Newman–Keuls post hoc test. For all procedures, *P*-values of < 0.05 were considered statistically significant.

Results

CSE-induces ETB and ETA up-regulation is prevented by bosentan.

In vitro exposure of HPASMCs to CSE elicited a dose and time-dependent increase of the ETA and ETB protein and mRNA expression (Figure 1A and 1B) reaching a peak value at CSE 10% after 24 h of stimulation. Based on these results we selected CSE 10% for 24h as the set stimulation condition for later studies. Pre-incubation of HPASMCs with bosentan (10nM-10μM) dose-dependently prevented ETA and ETB protein up-regulation (Figure 1C). The selective ETB antagonist BQ788 prevented the CSE-induced ETB up-regulation at 1μM and 10μM concentrations (Figure 1D), whilst no effect was observed on CSE-induced ETA up-regulation (Figure 1D). In contrast, the selective ETA antagonist BQ123 successfully prevented ET_AR up-regulation at 100nM-10μM and ET_BR up-regulation at 1μM and 10μM (Figure 1E). Studies performed on mRNA expression showed the same results as those observed for protein expression (Figure 1F and 1G).

CSE-induced ETB and ETA expression is partially mediated by ERK1/2, RhoA-GTP and intracellular ROS downstream pathways as well as by an autocrine ET-1 feed-forward mechanism.

Incubation of HPASMCs with the ERK1/2 inhibitor PD98059 (10μM), the Rho-kinase inhibitor Y27632 (10μM) or with the antioxidant NAC (1mM) effectively prevented ETB and ETA protein over-expression induced by CSE (Figure 2A). Likewise, HPASMCs incubation with mAb-ET-1 (10μg/ml) also suppressed CSE-induced ETB and ETA over-expression (Figure 2A). In other experiments ET-1 (10nM) addition (24h) increased ETB and ETA protein expression (Figure 2B). Bosentan (10μM), BQ123 (10μM), PD98059, Y27632 or NAC partially prevented the ET-1-induced ETB and ETA protein expression whilst BQ788 (10μM) only prevented ETB over-expression (Figure 2B). These results were in keeping with ETB and ETA gene expression (Figure 2C). Interestingly, CSE 10% (24h) significantly increased ET supernatant levels (Figure 2D; $P<0.05$ *vs*. control) which was prevented by bosentan, BQ788, BQ123, PD98059, Y27632 and NAC (1mM) (Figure 2D).

CSE-induced intracellular ROS, ERK1/2 phosphorylation and RhoA-GTP activation is prevented by bosentan.

CSE increased by ~2.73-fold the intracellular fluorescence intensity derived from DCF formation after 24h. Bosentan and BQ123 significantly reduced the CSE-induced ROS formation by respectively ~1.17-fold and ~1.5-fold whilst BQ788 did not reach a significant reduction (~2.23-fold) (Figure 3A and 3B) over control group. Furthermore, CSE increased the ERK1/2 phosphorylation and RhoA-GTP activation after 24h which was effectively prevented by bosentan, BQ123 and to a lesser extent by BQ788 (Figure 3C and 3D).

Bosentan prevents the CSE-induced HPASMC proliferation.

CSE increased cell proliferation by ~2-fold (Figure 4A). Bosentan inhibited cell proliferation whilst BQ123 and BQ788 reduced proliferation by ~1.31 and ~1.6 over control respectively (Figure 4). Moreover, PD98059, Y27632 and NAC (1mM) also prevented cell proliferation. Since it has been shown that CSE increases ET supernatant levels, we selectively blocked ET-1 with mAb-ET-1 which subsequently reduced cell proliferation to basal levels (Figure 4A). Since HPASMC proliferation *in vivo* is mainly mediated by growth factors such as platelet-derived growth factor (PDGF), we treated cells with CSE in presence or absence of human recombinant PDGF-BB at 10ng/ml. PDGF-BB increased cell proliferation by 3.1-fold in absence of CSE and 3.5-fold in presence of CSE. Bosentan, BQ123 and BQ788 inhibited cell proliferation induced by CSE and PDGF-BB by 1.6, 1.9 and 2.1-fold respectively (Figure 4B).

HPASMC exposure to CSE increases the ET-1-induced $[Ca^{2+}]_i$ and cell contraction.

HPASMCs exposed to CSE 10% were subject to an acute increase in ET-1 (10nM)-induced $[Ca^{2+}]_i$ with a peak value of 493±46 nM *vs.* 274±34 nM for untreated cells (control) (Figure 5A and 5B; $P<0.05$). Bosentan, BQ788 or BQ123 added before and during CSE exposure reduced the acute ET-1-induced peak increase in $[Ca^{2+}]_i$ to 304±41 nM, 395±36 nM and 343±32 nM respectively (Figure 5A and 5B; $P<0.05$ *vs.* CSE alone). ET-1 (10 nM) increased cell contraction and was significantly higher in those cells exposed to CSE (10%) *vs*. unexposed cells (Figure 5C; $P < 0.05$). Cells pre-treated with CSE in the presence of bosentan, BQ788 or BQ123 were less susceptible to ET-1-induced cell contraction as cell contraction was near to control levels (Figure 5C).

CSE exposure increases ET-1-induced human small intrapulmonary artery contraction which is prevented by bosentan.

Lung cut slices incubated with CSE showed an increase of ETB protein expression which was significantly prevented by bosentan, BQ788 and BQ123 whilst CSE-induced ETA protein expression was only prevented by bosentan and BQ123 (Figure 6A). These results were qualitatively reproduced by immunofluorescence experiments (Figure 6B). In this regard, CSE exposure increased the ETB fluorescence intensity in endothelial cells and smooth muscle cells. ETA fluorescence intensity was augmented only in the area of smooth muscle cells. Cut lung slices pre-treated with bosentan showed lower fluorescence intensity for both, ETB and ETA (Figure 6B). Precision lung cut slices pre-treated with CSE 10% increased vascular sensitivity to ET-1. The calculated log EC_{50} for CSE treated group was -9.36±0.32 M versus -8.47±0.16 M in controls (Figure 6C and 6D; $P < 0.05$). Cut lung slices exposed to CSE in presence of bosentan, BQ788 or BQ123 significantly increased the ET-1 log EC_{50} to -8.76±0.3 M, -8.73±0.17 M and -8.64±0.24 M respectively. CSE also increased the ET-1 *E*max contraction *vs*. control group (184±5.7 *vs.* 136±3.6; Figure 6F; $P<0.05$) which was attenuated by bosentan, BQ788 or BQ123 pre-treatments to ET-1 *E*max of 127.8± 4.3, 160.9±3.6 and 145.8±4.3 respectively (Figure 6F). On the other hand, pulmonary arteries from smokers and COPD patients showed an increase in ETA and ETB protein expression *vs.* non-smoker patients (Figure 7; $P<0.05$).

Discussion

The relevance of this study is based in two main assumptions: 1) Chronic cigarette smoke is responsible of pulmonary vascular remodeling and pulmonary hypertension

development along the COPD progression [17]; 2) Endothelin system is directly implicated in pulmonary remodeling of COPD patients [18]. Results observed in this study showed that ETRs antagonism could attenuate cigarette smoke-induced HPASMC proliferation and tension as well as small intrapulmonary artery tension by means of ETR and ET down-regulation, thus suggesting that bosentan could be useful to treat certain forms of pulmonary hypertension in smokers with COPD as suggested recently [19, 20]. Furthermore, we provide a novel mechanism of action by which cigarette smoke increases ETR expression through a feed-forward mechanism mediated by ET release and by the activation of ERK1/2, RhoA-GTP, and intracellular ROS. All these intracellular pathways were attenuated by bosentan.

Whether our results are applicable to *in vivo* cigarette smoke inhalation depends on the assumption that cigarette smoke components reach the vascular bed of smaller pulmonary arteries. Several observations strength this assumption: 1) Cigarette smoke components are rapidly taken up into the bloodstream during smoking (around one minute) [21], suggesting rapid equilibration across the gas exchange surface. 2) Many smoke components are highly water soluble such as peroxynitrite, which is considered a potent remodeling agent in pulmonary arteries [22], allowing easy solution into the alveolar lining and interstitial fluid. 3) Respiratory gas exchange has been demonstrated in pulmonary vessels as large as 3 mm [23], suggesting that smoke might have access to smaller pulmonary arteries. In this sense, we obtained same results in cells obtained from ~3mm arteries than in small intrapulmonary arteries of 100µm of internal diameter (precision cut lung slices experiments). 4) Smoke components are present in recirculating blood for several hours after smoking [24] allowing continued exposure to the lung vascular bed during recirculation.

The concentration of smoke components around the vascular bed is difficult to estimate. In this study we used cigarette smoke extract at 2.5% to 10% concentration which may correspond approximately to the exposure associated with smoking 0.5 to 2 packs per day as previously outlined [25], thus, our experiment probably estimated the biologic significance of smoking habit.

The ET system appears to have a large impact on the initiation and progression of lung vascular remodeling. In fact, exhaled breath condensate and circulating ET-1 levels are increased in COPD patients with PH and both are correlated with pulmonary systolic pressure [5]. Furthermore, animals exposed to CS show an increase in basal ET-1 levels and vascular contractility which may contribute to the pulmonary pathophysiology associated with CS [26]. Recent studies performed on rat arteries originating from various tissues, such as brain, mesentery and kidney, conclude that CSE up-regulates ETB and ETA expression by a mechanism which implicates the activation or phosphorylation of ERK1/2, p38, JNK, PKC and NF-κB [9-11]. However, currently no data about the effect of CS on ETR expression in human pulmonary arteries is available. In this work we have observed for the first time that the CSE-stimulated increase in ETB and ETA protein and gene expression can be counteracted by dual ETR antagonist bosentan as well as by the selective ETA antagonist BQ123. In contrast, the selective ETB antagonist, BQ788, only prevented ETB over-expression. This process was explained, in part, by a feed-forward mechanism mediated by ET release. In this regard, CSE was able to increase ET supernatant levels that were consequently suppressed by bosentan and BQ123 and to a lesser extent by BQ788. These results are apparently in contrast with previous reports [27], where the exposure of the selective ETB antagonist BQ788, and the dual ETB/ETA antagonist bosentan, increased ET-1 mRNA in endothelial cells. This effect was explained because extracellular levels of ET-1 are

cleaning *via* endocytosis of ETB, thus, its blockade impedes extracellular ET-1 cleaning inducing ET-1 up-regulation. However, results observed in this work were obtained in a totally different context of cigarette smoke extract exposure. In this regard, cigarette smoke increases ET release in endothelial cells and airway smooth muscle cells as previously mentioned [28, 29]. In this study, we observed that the cigarette smoke-induced ET release in HPASMCs was mediated by a mechanism including reactive oxygen generation, ERK1/2 phosphorylation and RhoA-GTP activation. This same mechanism has been also observed by our group in pulmonary artery endothelial cells [13]. Because blocking ETR is able to attenuate the cigarette smoke-induced ROS, ERK1/2 phosphorylation and RhoA-GTP activation, it is reasonable to assume that ETR antagonism inhibits cigarette smoke-induced ET release.

As occurred with CSE, ET-1 incubation was also able to up-regulate ETB and ETA expression in HPASMCs which in turn confirms similar results observed in HPAECs [13]. Since endothelial cells are the main reservoir of ET-1, one could hypothesize that cigarette smoke increases ET-1 in endothelial cells which could interact with HPASMCs increasing ETR expression. So, direct effect of ET-1 on cigarette smoke-induced ETR over-expression in HPASMCs can not be ruled.

It is interesting to note that ET-1 stimulation induced a higher ETA expression than CSE (see Figure 1A *vs.* Figure 2C). Since ETA is mostly induced by ET-1, it may be presumed that the impotence of BQ788 in preventing CSE-induced ETA expression may be due to its low effect on the CSE-induced ET release.

It is known that intracellular ROS may activate several intracellular pathways such as PKC, different MAPKs such as ERK1/2, p38 and JNK, as well as transcription factors such as NF-κB [30]. In fact, recent reports have shown that all these pathways are involved in CSE-induced ETB and ETA expression in rat basilar arteries [9, 10].

Furthermore, both CSE and ET-1 activate NADPH-oxidase complex to produce intracellular ROS [31, 32]. Interestingly, both the CSE oxygen specie H_2O_2 and ET-1-induced intracellular ROS are mediated by the activation of ETA since it has been shown that BQ123 inhibits them in foetal PASMCs [32]. In this work, the antioxidant NAC, bosentan and BQ123 attenuated the CSE-induced ROS, ETB and ETA over-expression. Contrastingly BQ788 did not influence the levels of ROS and ETA induced by CSE, so these results may explain, in part, the deficiency of BQ788 in affecting CSE-induced ETA expression. On the other hand, ERK1/2 inhibitor PD98059 was sufficient in preventing the CSE and ET-1-induced ETB and ETA expression which is according to previous reports in animal models [9, 11]. In this regard, bosentan, BQ123 and to a lesser extent BQ788 prevented ERK1/2 phosphorylation thus highlighting the role of ERK1/2 on CSE-induced ETR up-regulation.

It is known that smokers and COPD patients display increased levels of expression in RhoA-GTP as well as in the Rho-kinase downstream factor which is involved in endothelial dysfunction, vascular contractility and remodeling [33]. As we previously found in HPAECs [13], CSE's ability in up-regulating RhoA-GTP activity was preventable by bosentan, BQ123 and to a lesser extent by BQ788 treatment. Moreover, the Rho-kinase inhibitor Y23670 prevented the CSE and ET-1-induced ETB and ETA up-regulation which implicate RhoA-GTP activation in the process of ETR expression.

During the course of pulmonary vascular remodeling, the PASMC proliferation contributes to intimal thickening. Previously, studies have positively correlated CS to vascular SMC proliferation [34] by a mechanism involving ERK1/2 activation. In this study we observed that bosentan, BQ123 as well as BQ788 prevented HPASMC proliferation in a process involving both forms of ETR. Coupled with this, HPASMC

proliferation was mediated by the activation of intracellular ROS, ERK1/2 and Rho-kinase as well as by the autocrine ET action.

In other experiments it was reached that CSE exposure significantly increases acute ET-1-induced $[Ca^{2+}]_i$ and cell contraction. Further research revealed, according to CSE-induced ETA and ETB expression, the inhibitory efficacy of bosentan, BQ123 and BQ788 on these CSE derived outcomes.

Based on these *in vitro* results, we attempted to translate the effect of CSE on human small intrapulmonary arteries in a precision lung cut slice model. It is known that human pulmonary artery vascular remodeling occurs on small resistant-type intrapulmonary vessels (less than 3 mm) and precapillary arteries (around 20μm of internal diameter) which form part of the pulmonary vascular bed responsible for the pressure elevation observed in PH [3]. In the present work precision lung cute slices from small intrapulmonary arteries over-expressed ETA and ETB in vascular SMC and endothelial cells secondary to CSE exposure. Bosentan and to a lesser extent BQ123 inhibited ETR up-regulation whilst BQ788 only attenuated ETB which is in agreement with the *in vitro* cell data. An increase in pulmonary artery contraction secondary to acute ET-1 was an outcome of ETR up-regulation and was suppressed by bosentan and to a lesser extent by BQ123 and B788. These results may be considered as an approximation model of the *in vivo* conditions since we found that isolated pulmonary arteries from smokers and COPD patients up-regulates ETB and ETA.

Beside novel mechanistic pathways studied in this work, we are aware of the study limitations. First: we have conducted an *in vitro* acute (24 h) model of cigarette smoke exposure, and progressive worsening of pulmonary hemodynamic in humans are induced only by chronic cigarette smoke exposure over many years. Second: *in vitro* studies are not always representative of the *in vivo* findings since a number of

substances are released by cigarette smoke and counteracted with different cell types at the same time, so, results observed in isolated HPASMCs could be different *in vivo.* Third: although we have found ETRs over-expression in pulmonary arteries from smokers and COPD patients, none of them showed pulmonary hypertension, so whether COPD patients with pulmonary hypertension have ETR up-regulation or whether bosentan attenuates ETR up-regulation *in vivo* remains unknown.

The results of this study indicate that the dual ETR antagonist effectively decreased the ETR over-expression elicited by CSE in HPASMCs and small intrapulmonary arteries. This direct inhibitory effect could explain the beneficial effects of bosentan in certain forms of disproportionate pulmonary hypertension in COPD patients.

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Conflict of Interest: None declared.

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Figure legends

Figure 1

Cigarette smoke increases endothelin receptor A and B.

A) CSE dose-dependently and (B) time-dependently increased ETB and ETA protein and mRNA expression in HPASMCs. C) Bosentan, (D) BQ788 and (E) BQ123 dose-dependently prevented the CSE-induced ETB protein expression whilst only bosentan and BQ123 dose-dependently prevented ETA expression. Results are the mean±SEM of three different experiments per condition. **P*<0.05 compared with respective control condition. #*P*<0.05 compared with CSE group. CSE, cigarette smoke extract; ETA, endothelin receptor A; ETB, endothelin receptor B; HPASMCs, human pulmonary artery smooth muscle cells.

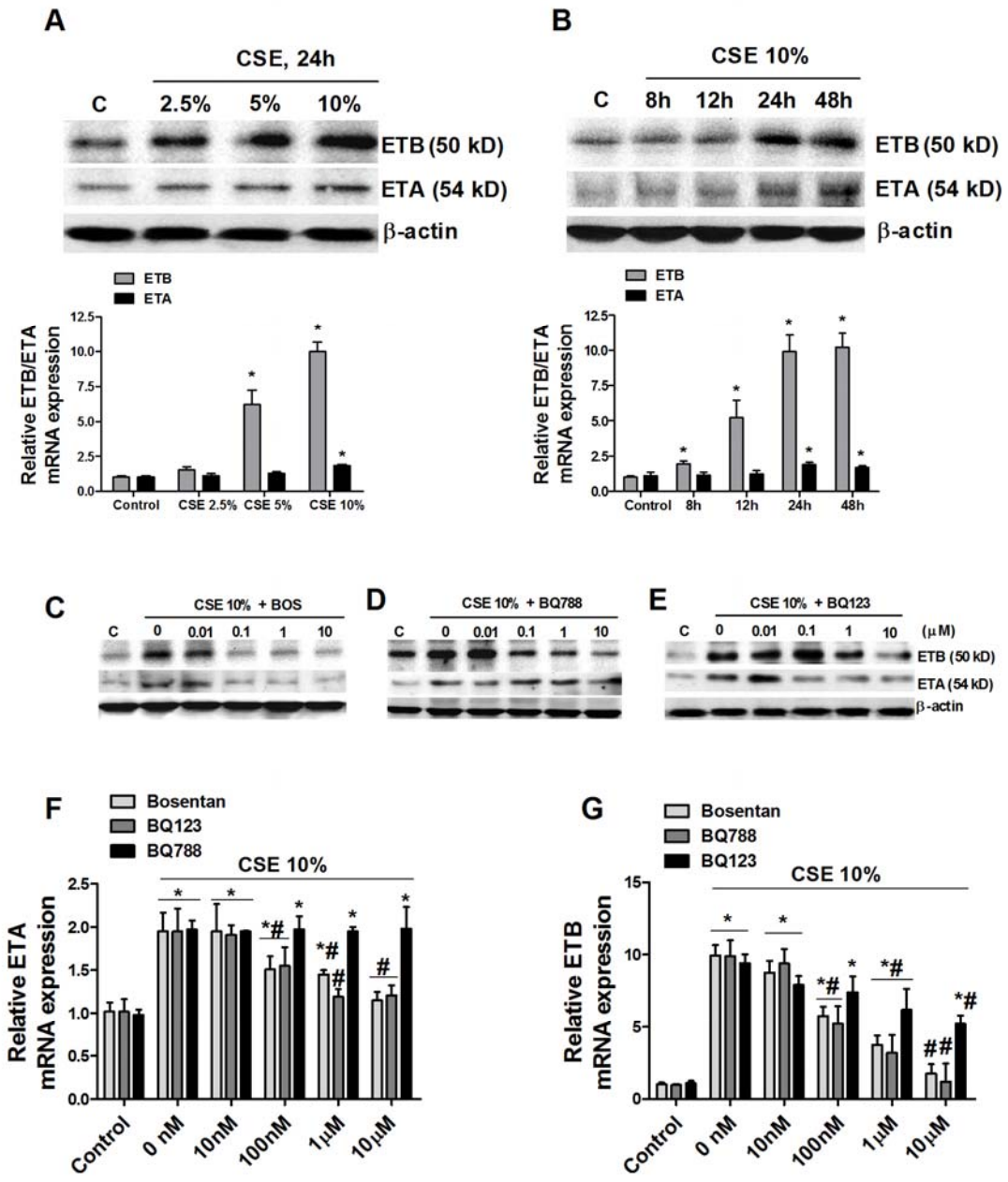

Figure 1

B)

Figure 2

Cigarette smoke increases endothelin recptor A and B by a mechanism which implicates intracellular ROS, ERK1/2, RhoA-GTP and autocrine endothelin feed-forward mechanism.

A) The increase of ETA and ETB induced by CSE 10% (24h of incubation) was prevented by PD98059 (10μM), Y27632 (10μM) or NAC (1mM) in HPASMCs. Incubation with mAb-ET-1 (10μg/ml) 30 min before CSE addition also prevented ETA and ETB up-regulation unlike the negative isotype IgG1 control. B and C) ET-1 10nM incubation for 24 h significantly increased ETA and ETB protein (B) and mRNA (C) expression which was prevented by bosentan (10μM), BQ123 (10μM), PD98059, Y27632 and NAC, whilst BQ788 (10μM) only prevented ETB but not ETA expression. D) CSE 10% increased ET release which was attenuated by bosentan, BQ123, BQ788, PD98059, Y27632 and NAC. Results are the mean±SEM of three different experiments per condition. **P*<0.05 compared with respective control condition. #*P*<0.05 compared with CSE group. CSE, cigarette smoke extract; ETA, endothelin receptor A; ETB, endothelin receptor B; HPASMCs, human pulmonary artery smooth muscle cells.

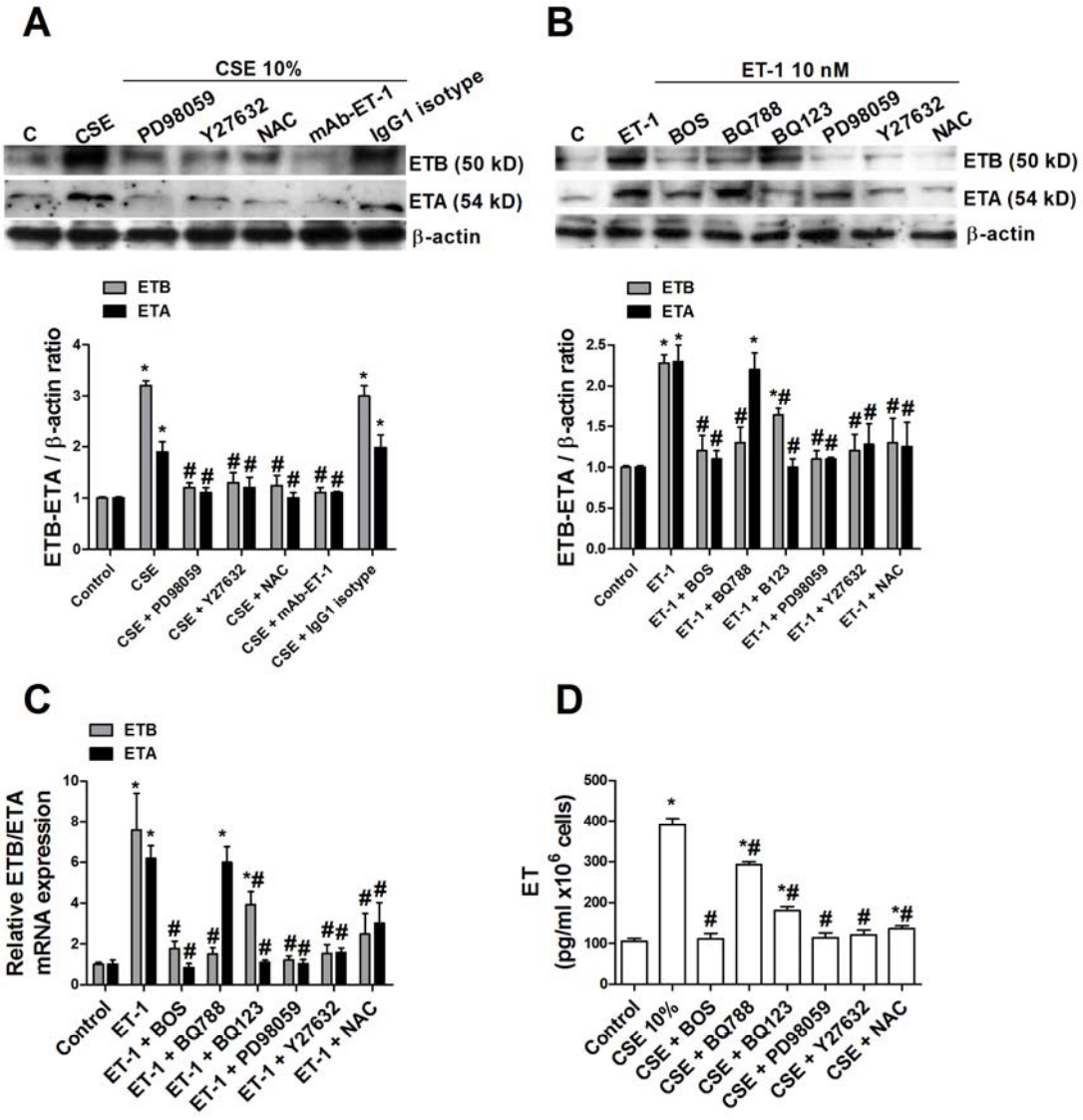

Figure 2

B)

Figure 3

Endothelin receptor antagonism attenuates the CSE-induced ROS, pERK1/2 and RhoA-GTP activation.

A and B) CSE 10% increases intracellular ROS generation after 24h of exposure as assessed by DCF fluorescence intensity in HPASMCs. The CSE-induced ROS generation was suppressed by bosentan (10μM) and by BQ123 (10μM) but not by BQ788 (10μM). Representative intracellular ROS fluorescence images are presented in

panel A. C) CSE 10% elicited ERK1/2 phosphorylation after 24h of exposure which was suppressed by bosentan, attenuated by BQ123 and, to a lesser extent, by BQ788. D) CSE 10% increased RhoA-GTP activation which was also suppressed by bosentan, attenuated by BQ123 and, to a lesser extent, by BQ788. Results are the mean±SEM of three different experiments per condition. **P*<0.05 compared with respective control condition. #*P*<0.05 compared with CSE group. CSE, cigarette smoke extract; DCF, 2′, 7′-dichlorofluorescein; HPASMCs, human pulmonary artery smooth muscle cells. RFU, relative fluorescence units; ROS, reactive oxygen species. Scale bar of images: 20μm.

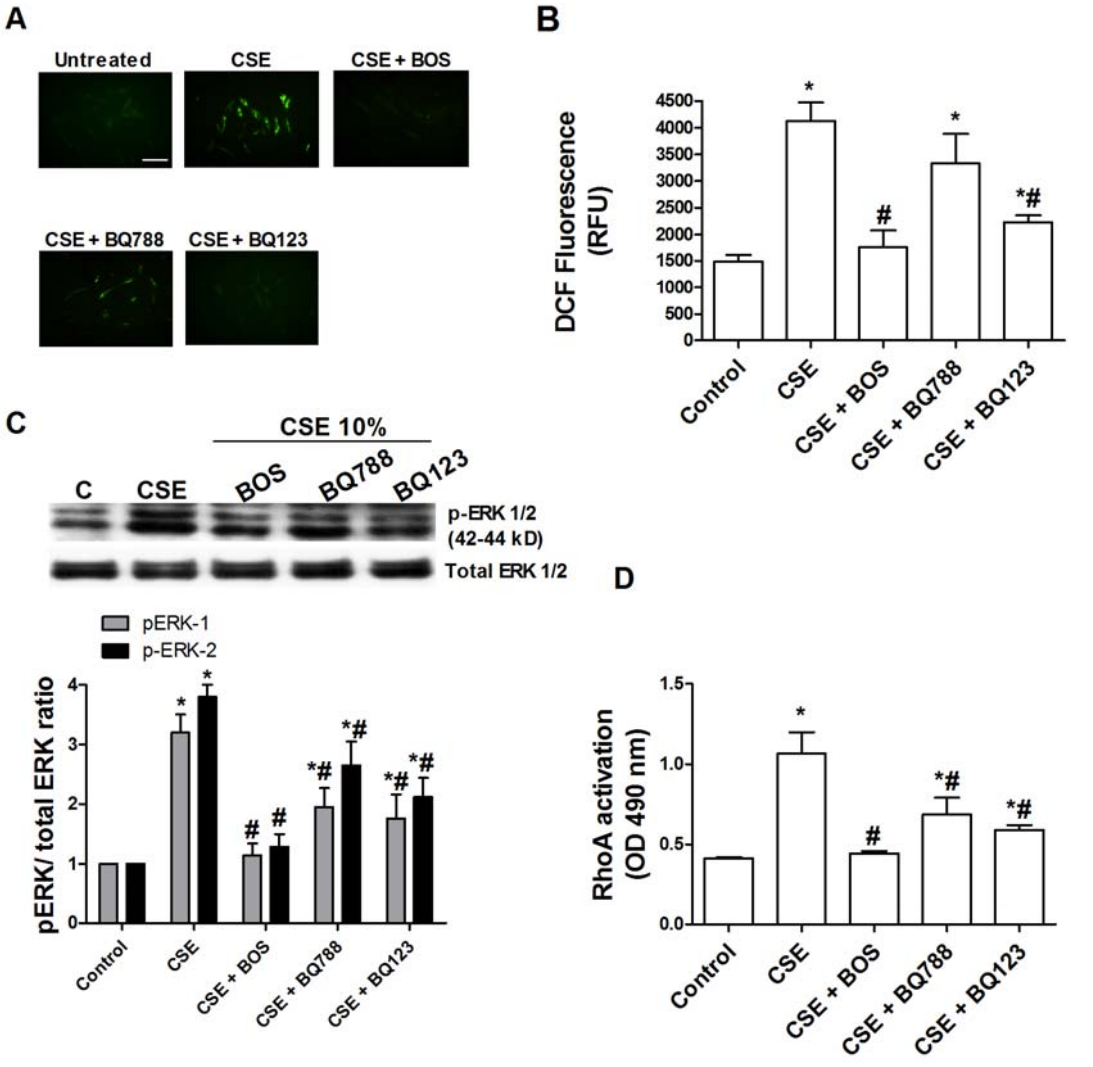

Figure 3

Figure 4

Cigarette smoke increases pulmonary artery smooth muscle cell proliferation.

A) CSE 10% increased HPASMCs proliferation after 24h of exposure. Bosentan (10μM), BQ123 (10μM), BQ788 (10μM), PD98059 (10μM), Y27632 (10μM), NAC (1mM) and mAb-ET-1 (10 μg/ml) effectively prevented the CSE-induced HPASMC proliferation. B) PDGF at 10ng/ml increased HPASMC proliferation by 3.1-fold. In presence of CSE 10%, PDGF (10ng/ml) increased HPASMC proliferation by 3.5-fold which was attenuated by Bosentan (10μM), BQ123 (10μM) and BQ788 (10μM).

Results are the mean±SEM of three different experiments per condition. **P*<0.05 compared with respective control condition. #*P*<0.05 compared with CSE group. δ*P*<0.05 compared with CSE plus PDGF group. CSE, cigarette smoke extract; HPASMCs, human pulmonary artery smooth muscle cells; NAC, N-acetyl-L-cysteine; mAb-ET-1, monoclonal antibody against endothelin 1; PDGF, platelet-derived growth factor.

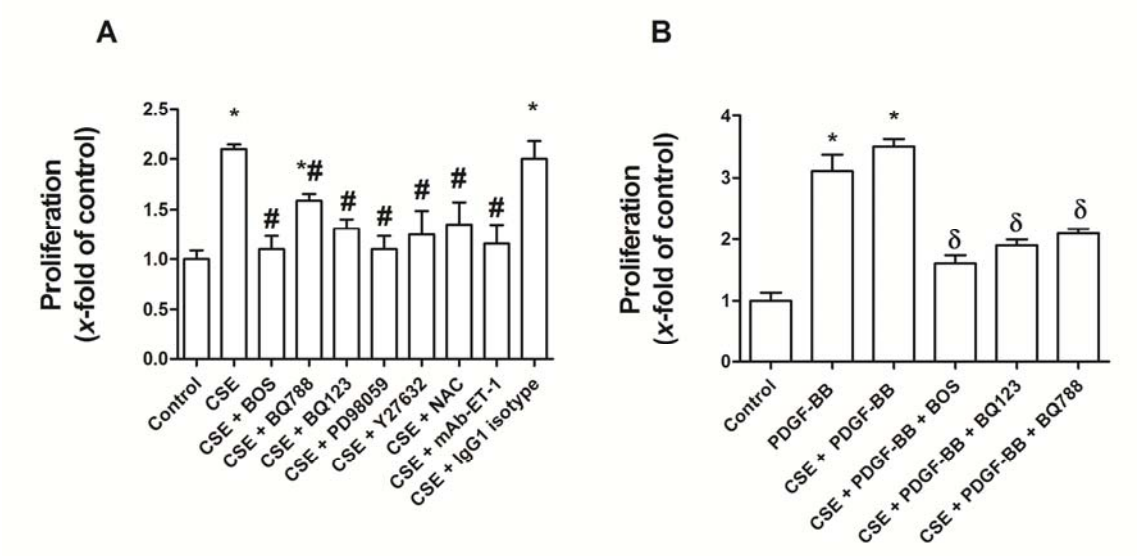

Figure 5

Cigarette smoke sensitizes pulmonary artery smooth muscle cells to acute ET-1-induced intracellular calcium and cell contraction.

HPASMCs were exposed to CSE10% in presence or absence of bosentan (10μM), BQ123 (10μM) or BQ778 (10μM) for 24 h. A and B) cells were washed three times with PBS and incubated with Fura 2AM for 30 min. Then cells were stimulated with acute ET-1 (10 nM). Values are show as $[Ca^{2+}]_i$ nM in 12 cells per experiment in a total of four experiments per condition. C) Disks with cultured HPASMCs were placed in the microscope and cells imaged with bright-field illumination. The graph C shows the time course of contractile response of HPASMCs challenged with ET-1 (10 nM). Values are expressed as total force exerted by the cell on the substrate in a total of 6 cells per condition. Results are the mean ± SEM of n experiment per condition.*$P < 0.05$ versus control; #$P < 0.05$ versus CSE 10%.

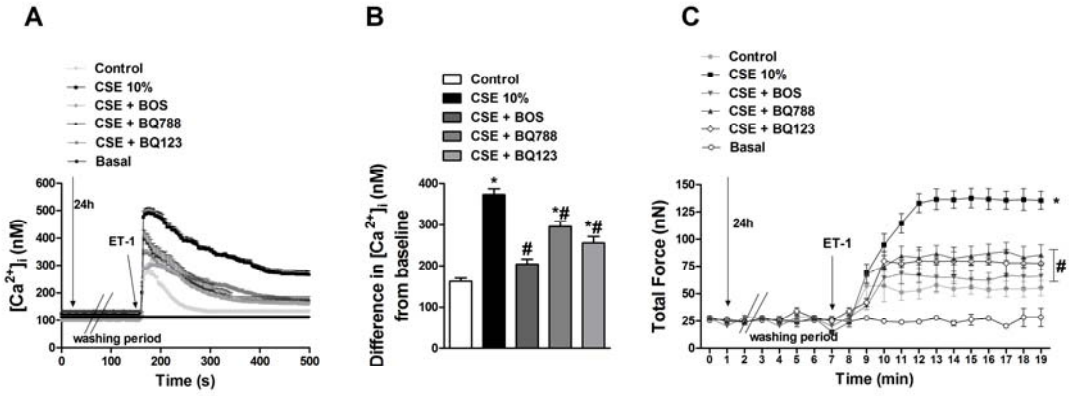

Figure 5

Figure 6

Cigarette smoke increases endothelin receptor A and B expression in human small intrapulmonary arteries which increase pulmonary vascular contractility in response to acute ET-1.

Small human intrapulmonary precision cut slices were exposed to CSE 10% in presence or absence of bosentan (10μM), BQ123 (10μM) or BQ788 (10μM) for 24h. A) Western blot was performed to measure the ET_AR and ET_BR protein expression. B) Cut slices were fixed and inmunofluorescence was performed for ETA and ETB with secondary rhodamine antibody. Images for IEL and EEL were assessed by green autofluorescence. Scale bar: 100 μm. C) Representative images of the time-course human small pulmonary artery contraction in response to ET-1 under visible video-microscope. Scale bar: 250μm. D, E and F) Concentration-dependent ET-1 contraction curves in two slices per condition of a total of three different patients per condition. Results are the mean ± SEM of A, B) three, D, E, F) 2 slices x 3 patients different experiments per condition.*P < 0.05 versus control; #P < 0.05 *versus* CSE 10%. CSE, cigarette smoke extract; EEL, external elastic lamina; IEL, internal elastic lamina; ETA, endothelin receptor A; ETB, endothelin receptor B.

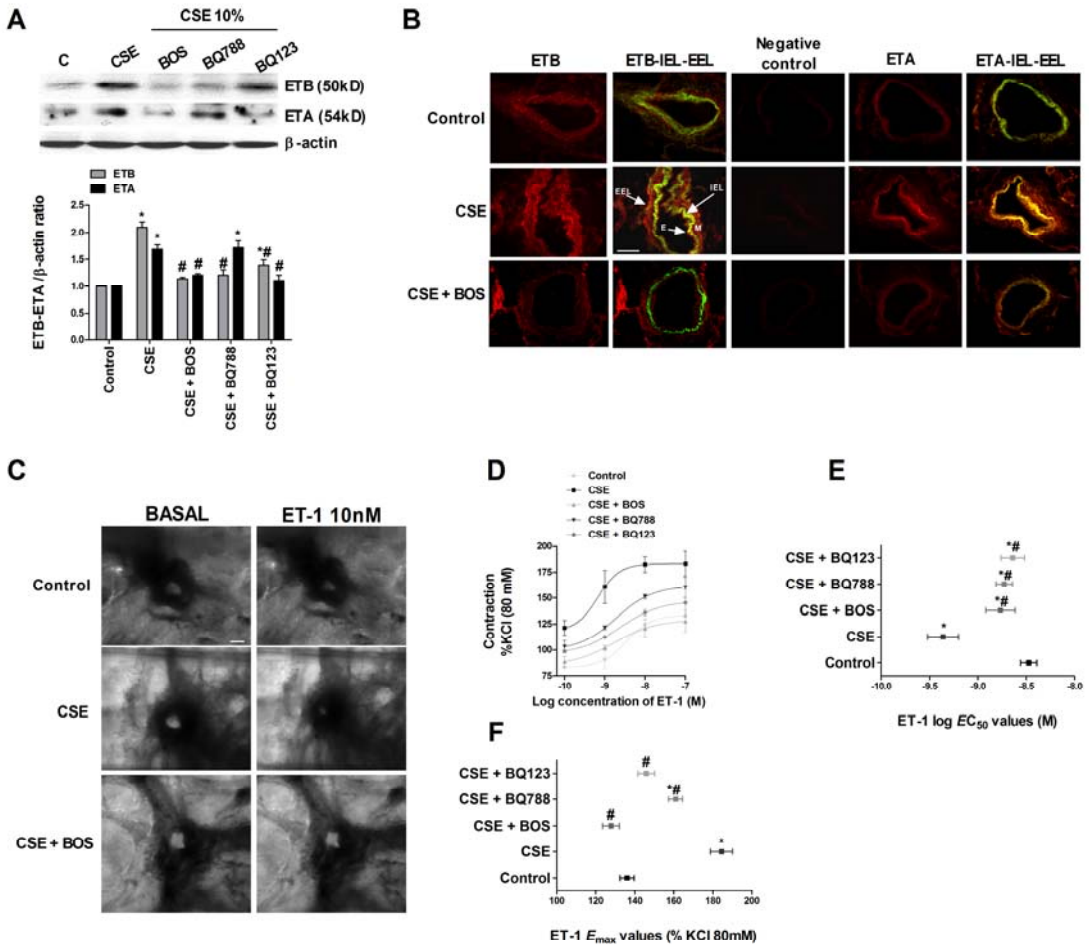

Figure 6

Figure 7

Endothelin receptor A and B are up-regulated in pulmonary arteries from smokers and COPD patients.

Pulmonary arteries from non-smokers, smokers and COPD patients were carefully isolated from non-traumatic human lung tissue of surgical specimens. Total protein was extracted and proved for ETA and ETB antibodies by western blot. Picture represents the expression of ETA, ETB and the housekeeping β-actin from 6 non-smoker, 7 smokers and 8 COPD patients. Graph represents values of densitometry of ETA, ETB protein expression relative to β-actin and normalised to non-smoker expression. Results

are mean±SEM of densitometries of one western blot per patient. *P<0.05 from non-smokers.

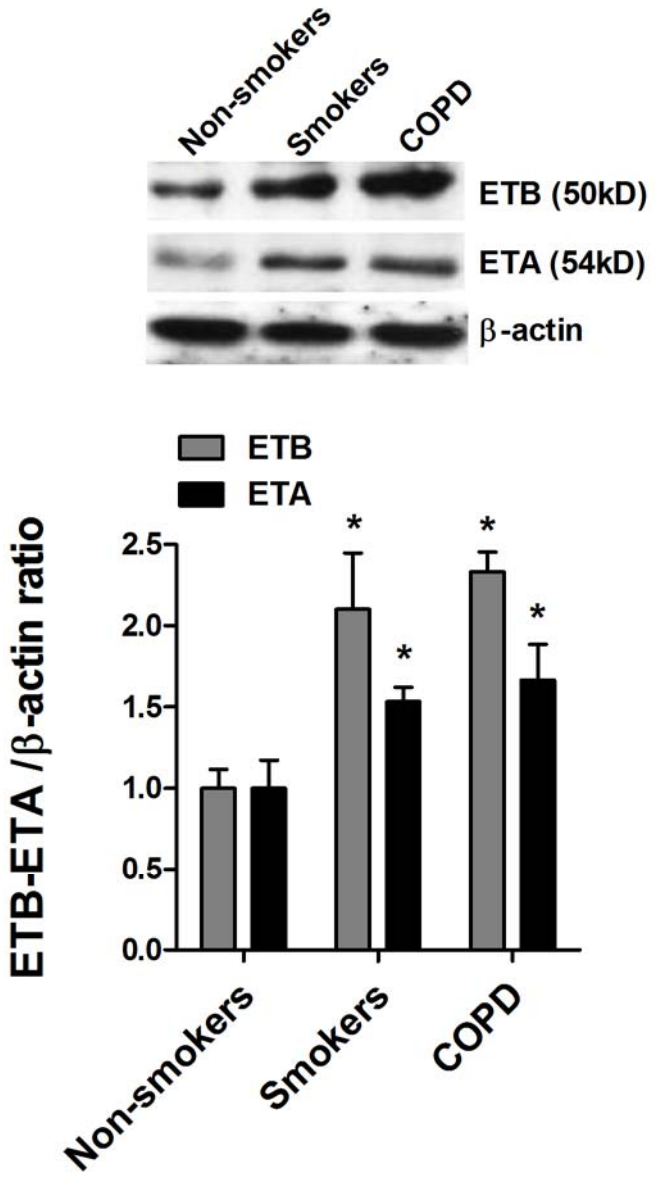

Figure 7

Table 1. Clinical features**.** FEV1: forced expiratory volume in one second; FVC: forced vital capacity; TLC: total lung capacity; PaO_2: oxygen tension in arterial blood; $PaCO_2$: carbon dioxide tension in arterial blood; Pack-yr = 1 year smoking 20 cigarettes-day.

	Non-smokers (n=6)	Smokers (n= 7)	COPD (n= 8)
Age, yr	66±5	68±7	67±9
Tobacco consumption, pack-yr	0	30±3	40±8
FEV1, % pred	92±3	90±6	68±8
FVC, % pred	94±4	93±8	87±6
FEV1/FVC %	89±5	87±7	63±5
TLC %pred	90±6	88±3	97±4
PaO_2, mmHg	90±8	85±7	84±6
$PaCO_2$ mmHg	37±6	39±3	41±3