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Cigarette smoke exposure up-regulates endothelin receptor B in human pulmonary artery endothelial cells: molecular and functional consequences

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Cigarette smoke exposure up-regulates endothelin receptor B in human pulmonary artery endothelial cells: molecular and functional consequences

J Milara[1, 6], JL Ortiz[2], G Juan[3], R Guijarro[4], P Almudever[1, 6], M Martorell[5], EJ Morcillo[2, 6,7] and J Cortijo[1, 2, 6]

[1]Research Unit, University General Hospital Consortium, Valencia, Spain. Av. tres cruces s/n. E-46014.

[2]Department of Pharmacology, Faculty of Medicine, University of Valencia, Spain, Av. Blasco Ibanez 17, E-46010.

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

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

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

[6]CIBERES, Health Institute Carlos III, Valencia, Spain.

[7]Clinical Pharmacology Unit, University Clinic Hospital, Valencia, Spain. Spain, Av. Blasco Ibanez 17, E-46010.

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

Short running title: Cigarette smoke and endothelin B receptor

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Abstract

Background and purpose: Pulmonary arteries from smokers and COPD patients show abnormal endothelium-dependent vascular reactivity. We studied the effect of cigarette smoke extract (CSE) on endothelin receptor B (ET_B) expression in human pulmonary artery endothelial cells (HPAECs) and its role on endothelial dysfunction.

Experimental approach: ET_B expression was measured by real time RT-PCR, western blot and immunofluorescence. Cell contraction, intracellular Ca^{2+}, F/G actin, RhoA activity, MLC-phosphorylation, ET, NO, TxA_2 and ROS production were measured by traction microscopy, fluorescence microscopy, phalloidin fluorescence, colorimetric assay, western blot, ELISA and DCFDA fluorescence respectively.

Key results: CSE dose-dependently increased ET_B expression in HPAECs after 24 h of incubation. The CSE-induced ET_B expression was attenuated by bosentan, the ET_B antagonist BQ788, the Rho kinase antagonist Y27632 and the antioxidant N-acetylcisteine (NAC). Furthermore, mAb-ET-1 effectively prevented the CSE-induced ET_B over-expression. In this regard, ET-1 dose-dependently increased ET_B expression after 24 h of exposure mimicking the effect of CSE. Cellular functional consequences of CSE-induced ET_B over-expression were translated into a higher cell contraction, an increased intracellular Ca^{2+}, F/G actin, RhoA activity, MLC phosphorylation, TxA_2 and ROS production, and a decrease of NO in response to acute ET-1 (10nM). These effects were attenuated by bosentan, BQ788, Y27632 and NAC. **Conclusions and Implications:** CSE induces ET_B over-expression by a feed-forward mechanism mediated in part by the ET release, promoting HPAEC dysfunction which is partially attenuated by ET_B blockage, Rho kinase and ROS inhibition. These results may provide in vitro evidence of the use of bosentan in CS-related endothelial dysfunction.

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Keywords: pulmonary artery endothelial cells; endothelin receptor B; bosentan; cigarette smoke; endothelial dysfunction.

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Introduction

Cigarette smoke (CS) is the major pathogenic factor implicated in chronic obstructive pulmonary disease (COPD), and pulmonary hypertension (PH) develops in approximately 10% of smokers with COPD (Voelkel *et al.*, 2003).

Pulmonary arteries of COPD patients show abnormal endothelium-dependent vascular reactivity (Dinh-Xuan *et al.*, 1991; Peinado *et al.*, 1998). Furthermore, endothelial function of pulmonary arteries in healthy smokers lies between that in non-smokers and COPD patients, thereby indicating that endothelial dysfunction is present at the origin of the disease (Peinado *et al.*, 1998). The impairment of endothelial function results from changes in the expression and release of vasoactive mediators that also regulate cell growth (Wright *et al.*, 2004). Overall, these initial alterations may lead to persistent changes in the vascular structure and function that underlie the development of PH in COPD.

Many reports have shown the deleterious effect of CS on endothelial function (Milara *et al.,* 2010; Santos *et al.*, 2002; Wright *et al.*, 2004). Thus, CS induces the expression and release of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α among others (Milara *et al.,* 2010; Orosz *et al.*, 2007; Wright *et al.*, 2004). Furthermore, CS promotes endothelial cell contraction through the activation of RhoA and myosin light chain (MLC) kinase which in turn increases endothelial cell permeability that is an important component of the pathogenesis of PH (Bernhard *et al.*, 2005; Morrell *et al.*, 2009; Richens *et al.*, 2009). These effects are related with the CS-impaired nitric oxide (NO) release in endothelial cells, since the reactive oxygen species (ROS) derived from CS inactivate endothelial NO synthase (eNOS) through the interaction of Rho-kinase with eNOS (Sugimoto *et al.*, 2007). On the other hand, products contained in cigarette

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smoke decrease the vasodilators/antimitogenics NO and prostacyclin (PGI_2) and increase the release of vasoconstrictors/mitogenics, such as endothelin-1 (ET-1) and thromboxane A_2 (TxA_2) promoting endothelial dysfunction and vascular remodelling (Barua *et al.*, 2003; Nana-Sinkam *et al.*, 2007; Wright *et al.*, 2006).

ET-1 is a secreted peptide that signals through two transmembrane G protein-coupled receptors ET_A and ET_B. These receptors are both simultaneously expressed in all cell types studied, with one notable exception; only the ET_B is expressed on endothelial cells (Migneault *et al.*, 2005). In normal conditions endothelial ET_B elicit vasodilatation and anti-mitogenic effects through the release of NO and/or PGI_2 in pulmonary endothelial cells, and in a lesser extent releases ET-1 and TxA_2 (Galie *et al.*, 2004). Furthermore, pulmonary ET_B also mediates the pulmonary clearance of circulating ET-1 and the reuptake of ET-1 by endothelial cells (Dupuis *et al.*, 1996). At this respect, the role of ET_B on PH induced by hypoxia has been well established since endothelial cell-specific ET_B knockout mice present an exaggerated increase in right ventricular pressure during hypoxia (Kelland *et al.,* 2010). However, CS-induced PH in COPD patients has other implications different to the strictly hypoxemic found in primary PH. In this regard, it has been found a correlation between the number of inflammatory cells infiltrating the wall of pulmonary arteries with the enlargement of the intimal layer and endothelial dysfunction (Peinado *et al.*, 1999). Furthermore, it has been shown recently that systemic inflammation in COPD appears to increase the risk for developing PH in COPD in humans as well as in vitro and animal models of pulmonary artery remodelling related to tobacco smoke (Eddahibi *et al.*, 2006; Joppa *et al.*, 2006; Stenmark *et al.*, 2006). Interestingly, ET_A and ET_B receptors are upregulated in bronchial biopsies from COPD patients and ET-1 is upregulated in COPD patients with PH (Carratu *et al.*, 2008; Moller *et al.*, 1999) which suggest that changes in the ET

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signalling system may not be limited to an increased production of ET-1. In this regard, it has been shown recently that CS mediates the upregulation of ET_A and ET_B on rat bronchial and vascular smooth muscle cells, thus increasing their contractility (Granstrom *et al.*, 2006; Xu *et al.*, 2008). However no data is reported about the effect of CS on ET_B expression and its functional consequence on endothelial cells.

Based on this background we hypothesized that CS, the main agent involved in COPD and the consequent endothelial dysfunction, may affect ET_B expression on human pulmonary arterial endothelial cells (HPAECs), and that this variation could modify HPAEC function. Therefore, the use of dual ET_A-ET_B antagonist bosentan proposed as a therapeutic agent in some forms of PH in COPD (Barbera *et al.*, 2009) could prevent the effect of CS derived from ET_B expression *in vitro*.

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Material and methods

Unless stated otherwise, all reagents used were obtained from Sigma Chemical Co. (Madrid, Spain). Bosentan (provided by Actelion Pharmaceuticals Ltd), BQ788, ML-7 and N-acetylcysteine (NAC) were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock solutions. Several dilutions of the stocks were prepared, using cell culture medium. The final concentration of DMSO in the culture medium did not exceed 0.01% and had no significant pharmacological activity. ET-1 and Y27632 were dissolved in sterile water. Mouse monoclonal antibody against human ET-1 (mAb-ET-1) (Abcam; cat. nº ab20940) was used at 10 μg/ml concentration to suppress the effect of the ET-1 supernatant levels as previously outlined (Didier *et al.*, 2003). Nonspecific mouse IgG1 was used at the same concentration as control.

Isolation and culture of human pulmonary artery endothelial cells

Cellular experiments were performed always in human pulmonary artery endothelial cells (HPAECs) from non-smokers in order to better in *vitro* study of cigarette smoke. Segments of pulmonary artery (2-3 mm internal diameter) were dissected free from parenchyma lung tissue, cut longitudinally, and digested with 1% collagenase (Gibco, UK) in RPMI-1640 culture medium for 30 min at 37°C. The digestion was neutralized by adding RPMI 1640 supplemented with 20% foetal calf serum (FCS), and the homogenate was separated by centrifugation at 1100 rpm. The pellet was resuspended, and cells were cultured in EGM-2 endothelial culture medium supplemented with Single Quotes (Clonetics, UK), 10% FCS, 1% fungizone, and 2% streptomycin/penicillin.

The selection of HPAECs was performed as described previously (Hewett *et al.*, 1993; Ortiz *et al.*, 2009), modified to include the use of a commercially available Dynabeads CD31 endothelial cell kit (Dynal Biotech, Germany). Briefly, cells were trypsinized (0.25% trypsin), and the cell mixture was incubated with CD-31-coated Dynabeads for 30 min at 4°C with end-over-end rotation. After incubation, the HPAECs were collected using a magnetic particle concentrator (MCP-1; Dynal) and washed four times with cold phosphate-buffered saline (PBS)/bovine serum albumin (BSA). Clusters of purified HPAECs retained on the CD-31-coated Dynabeads were separately resuspended in EGM-2 full growth medium supplemented with 10% FCS, 1% fungizone, and 2% streptomycin/penicillin. The cells not retained on the CD-31-coated Dynabeads were discarded.

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Real time RT–PCR

Total RNA was isolated from cultured HPAECs by using TriPure® Isolation Reagent (Roche, Indianapolis, USA). Integrity of the extracted RNA was confirmed with

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Bioanalizer (Agilent, Palo Alto, CA, USA). The reverse transcription was performed in 300 ng of total RNA with TaqMan reverse transcription reagents kit (Applied Biosystems, Perkin-Elmer Corporation, CA, USA). cDNA was amplified with specific primers for ET_B and RhoA (pre-designed by Applied Biosystems, ET_B: cat. nº: Hs00240747_m1; RhoA: cat. nª: Hs00357608_m1) and GAPDH (pre-designed by Applied Biosystems, cat. nº: 4352339E) as a housekeeping in a 7900HT Fast Real-Time PCR System (Applied Biosystem) using Universal Master Mix (Applied Biosystems). Relative quantification of these different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using GAPDH as endogenous control (Applied Biosystems; 4352339E) and normalized to control group.

Transfection of siRNAs

Small interfering RNA (siRNA), including the scrambled siRNA control, were purchased from Ambion (Huntingdon, Cambridge, UK). RhoA-targeted siRNA (ID: s758) sense 5'-CACAGUGUUUGAGAACUAUtt-3' and antisense 5'-AUAGUUCUCAAACACUGUGgg-3' were designed from Ambion. The HPAECs were transfected with siRNA (50 nM) in serum and antibiotic free medium and over a period of 6 h, the medium was then aspirated and replaced with serum containing medium for a further period of 42 h before CSE experiments. The transfection reagent used was lipofectamine-2000 (Invitrogen, Paisley, UK) at a final concentration of 2µl/ml. The mRNA expression for RhoA transcript was determined by real time RT-PCR after 48 h post-silencing and compared with siRNA control at the respective time to determine silencing efficiency. Furthermore, RhoA protein expression was measured by western blot after 48 h of silencing using a commercial monoclonal mouse anti-human RhoA antibody (AbD serotec, UK, cat. nº MCA5312Z). Protein expression was

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3 referred to β-actin (1:1000, Sigma, USA) expression as internal control. All
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6 experiments were performed in triplicate.

Western blot

Western blot analysis was used to detect changes in ET_B (50 kD), MLC (18 kD) and RhoA (22 kD). Cells were scraped from a confluent 25-cm^2 flask and lysed on ice with a lysis buffer consisting of a complete inhibitor cocktail plus 1 mM ethylenediaminetetraacectic acid (Roche Diagnostics Ltd, West Sussex, UK) with 20 mM Tris base, 0.9% NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, and 1 μg ml^{-1} pepstatin A. The Bio-Rad assay (Bio-Rad Laboratories Ltd., Herts, UK) was used (following manufacturer's instructions) to quantify the level of protein in each sample to ensure equal protein loading. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to separate the proteins according to their molecular weight. Briefly, 20 μg proteins (denatured) along with a molecular weight protein marker, Bio-Rad Kaleidoscope marker (Bio-Rad Laboratories), was loaded onto an acrylamide gel consisting of a 5% acrylamide stacking gel stacked on top of a 10% acrylamide resolving gel and run through the gel by application of 100 V for 1 h. Proteins were transferred from the gel to a polyvinylidene difluoride membrane using a wet blotting method. The membrane was blocked with 5% Marvel in PBS containing 0.1% Tween20 (PBS-T) and then probed with a rabbit anti-human ET_B (1:1,000) antibody (polyclonal antibody, cat. nº: E9905, Sigma, UK), rabbit anti-human diphospho-MLC (Thr18/Ser19) and rabbit anti-human MLC (1: 1,000) antibodies (rabbit polyclonal antibodies, cat. nº: 3674 and 3672, Cell signaling), monoclonal mouse anti-human RhoA (1:1,000) antibody (AbD serotec, UK, cat. nº MCA5312Z) or β-actin (1:1,000) as control, followed by a peroxidase conjugated secondary (1:10,000) antibody. The

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enhanced chemiluminescence method of protein detection using enhanced chemiluminescence reagents, ECL plus, (Amersham GE Healthcare, Buckinghamshire, UK) was used to detect labelled proteins.

Preparation of cigarette smoke extracts solutions

Cigarette smoke extracts (CSE) were prepared as we previously reported (Milara *et al.,* 2010; Ortiz *et al.*, 2009). Briefly, the smoke of a research cigarette (2R4F; Tobacco Health Research, University of Kentucky, KY, USA) was generated by a respiratory pump (Apparatus Rodent Respirator 680; Harvard, Germany) through a puffing mechanism related to the human smoking pattern (3 puff/min; 1 puff 35 ml; each puff of 2 s duration with 0.5 cm above the filter) and was bubbled into a flask containing 25 ml of pre-warmed (37°C) EGM-2 medium. The CSE solution was sterilized by filtration through a 0.22-μm cellulose acetate sterilizing system (Corning, NY). The resultant CSE solution was considered to be 100% CSE and was used for experiments within 30 min of preparation. CSE 10% corresponds approximately to the exposure associated with smoking two packs per day (Su *et al.*, 1998). The quality of the prepared CSE solution was assessed based on the absorbance at 320 nm, which is the specific absorption wavelength of peroxynitrite. Stock solutions with an absorbance value of 3.0 ± 0.1 were used. To test for cytotoxicity from CSE, HPAECs 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 epifluorescence microscopy (Nikon TE200, Tokyo, Japan) in HPAECs using the Ca^{2+} indicator dye

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fura-2 as previously outlined (Dalli *et al.*, 2008). In brief, fluorescence of fura-2 acetoxymethyl ester (5μM)-loaded cells was measured by using continuous rapid alternating excitation (340 and 380 nm) and emission (510 nm) in a fluorescence spectrophotometer equipped with a xenon lamp (Spectramaster System, Perkin Elmer, Life Sciences, Cambridge, UK) and a CDD camera CoolSNAPfx photometrics (20 MHz, 1300x1030 pixel). The 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 outlined (Grynkiewicz *et al.*, 1985). The following formula was used to converse fluorescence signal into $[Ca^{2+}]_i$; $[Ca^{2+}]_i = \beta \cdot K_d$ ($R - R_{min}$ / $R_{max} - R$), where R is the ratio between the fluorescence (F_{340}/F_{380}) at 340nm and 380nm, β is the ratio of 380 nm intensities at zero Ca^{2+} over maximal Ca^{2+} and K_d is the dissociation constant at 224 nM. Background levels of fluorescence at each excitation wavelength were determined in cell free areas and substracted for each experiment. Minimum and maximum fluorescence intensities were obtained with the addition of Ca^{2+}-free solution with 10mM EDTA solution and the Ca^{2+} ionophore ionomycin 10μM in presence of 5mM $CaCl_2$ solution respectively as previously outlined (Cortijo *et al.,* 2010; Dalli *et al.*, 2008). The experiments performed on intracellular Ca^{2+} were designed to study the functional effect of the CSE-induced ET_B over-expression.

Actin staining

Cells were washed three times with phosphate-buffered saline (PBS) and fixed in a 3.7% formaldehyde-PBS solution for 10 min at room temperature. After two additional washes with PBS, cells were permeabilized with a solution of 0.1% Triton X-100 in

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PBS for 3–5 min and washed again with PBS. Phalloidin-tetramethylrhodamine isothiocyanate (0.2 μg/ml) and Alexa Fluor 488 DNase I conjugate (9 μg/ml) were used to localize F-actin and G-actin, respectively, as described by Cramer and coworkers (Cramer *et al.*, 2002). Fluorescent dyes were diluted on blocking solution (1% BSA and 0.025% saponin in PBS) and added to coverslips for 40 min at room temperature. After three washes with PBS, coverslips were mounted on a microscopy slide with mounting media (mowiol; Calbiochem, La Jolla, CA). F-actin-to-G-actin fluorescence ratio was quantified using fields containing >30 cells imaged with an inverted fluorescence microscope (Eclipse TE200, Nikon) and a 12-bit-resolution cooled-charge-coupled device camera (CoolSNAPfx photometrics) at x10 magnification. Time of image acquisition and image intensity gain were optimally adjusted and kept constant for all experiments with the software Metafluor® 5.0. F and G-actin cytoskeleton imaging was performed with an epifluorescence microscope ((Nikon TE200, Tokyo, Japan) at x40 magnification.

Cell contraction

Contraction of HPAECs in response to ET-1 was studied by traction microscopy as previously outlined (Cortijo *et al.,* 2010). Collagen-coated polyacrilamide gels with embedded fluorescent microbeads (200-nm diameter) were used. Gel disks with cultured HPAECs were incubated for 24 h in absence (control) or presence of CSE 10% alone or in combination (1 h before CSE) with bosentan, BQ788, ML-7 or Y27632. Then, gel disk with cultured HPAECs was placed in the microscope and cells imaged with bright-field illumination. After 5 min of baseline recording, ET-1 (10 nM) was added, and fluorescent images were acquired for an additional 10 min. Traction forces exerted by the cell on the substrate were computed from the displacement field of the

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gel substrate (Cortijo *et al.,* 2010). Measurements were taken in n=10 cells from different cell-gel samples for different conditions.

Rho Activity Assays

A commercially available, enzyme-linked immunosorbent assay (ELISA)-based RhoA activity assay (G-LISA; Cytoskeleton, Denver, CO) was used to measure the relative RhoA activity of serum-starved HPAECs after experimental treatments. Whole cell lysates were processed as per the G-LISA protocol using lysis buffer provided in the kit. The lysates were incubated in microwells to which the rhotekin binding domain peptide was bound, and active RhoA was detected using indirect immunodetection followed by a colorimetric reaction measured by absorbance at 490 nm.

ET, NO and TxA$_2$ production

ET was measured in HPAEC culture supernatants by enzyme immune assay kit (Cayman chemical, USA) according to the manufacturer's protocol. NO was measured as nitrites in HPAEC culture supernatant samples, using a commercially available nitric oxide assay kit (Calbiochem-Novabiochem, San Diego, CA) according to the manufacturer's protocol. TxA$_2$ was measured as TxB$_2$ (a stable metabolite of TxA$_2$) in HPAEC culture supernatants by enzyme immune assay kit (Cayman chemical, USA) according to the manufacturer's protocol.

DCFDA fluorescence measurement of reactive oxygen species.

The fluorogenic substrate 2´, 7´-dichlorofluorescein diacetate (DCFDA, Molecular proves, UK) is a cell-permeable dye that is oxidized to highly fluorescent 2´, 7´-dichlorofluorescein (DCF) by $O_2^{\cdot-}$ and H_2O_2, and can therefore be used to monitor

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intracellular generation of ROS. To quantify ROS levels, HPAECs were seeded to black walled, clear bottom 96 well plates, washed twice with PBS and incubated for 30 min with 50 μM DCFDA diluted in Opti-MEM with 10% FCS. At the end of the incubation period, the cells were again washed twice with PBS and stimulated with ET or CSE. Then, fluorescence was measured using a microplate spectrophotometer (Victor 1420 Multilabel Counter, PerkinElmer) at excitation and emission wavelengths of 485 and 528 nm, respectively, at 5 min intervals for a total of 45 min. Results were expressed as DFC fluorescence in relative fluorescence units (RFU) versus time (min).

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Immunofluorescence

HPAECs were washed three times with PBS and fixed (4% paraformaldehyde, 30 min, at room temperature). After another three washes with PBS, HPAECs were permeabilized (20 mM HEPES pH 7.6, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100), blocked (10% goat serum in PBS) and incubated with the primary antibody rabbit anti-human ET_B (1:100, cat. nº: E9905, Sigma) overnight at 4°C, followed by secondary antibody anti-rabbit rhodamine (1:100, Molecular Probes) and DAPI (2μg/ml) to mark nuclei (Molecular Probes, Leiden, The Netherlands). Cells were visualized by epifluorescence microscopy (×200; Nikon eclipse TE200 inverted microscope, Tokyo, Japan).

Experimental protocols

The experiments performed on cell contraction, intracellular Ca^{2+}, F/G actin, RhoA activity and MLC phosphorylation, NO and TxA_2 production and ROS production were designed to study the functional effect of the CSE-induced ET_B over-expression. To this respect HPAECs were treated with or without CSE for 24 h in presence or absence

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of bosentan or different pharmacological modulators (added 1 h before CSE). Then, HPAECs were washed three times with PBS and stimulated with acute ET-1 (10nM) for different times to mimic the lung microenvironment associated to PH where ET is highly elevated (Cacoub *et al.*, 1997; Holm *et al.*, 1996). At the end of the ET-1 stimulation, the above defined parameters where measured and compared to CSE-unexposed cells.

Statistics

Data are presented as mean ± SEM of n experiments. Statistical analysis of data was carried out by analysis of variance (ANOVA) followed by Bonferroni test (GraphPad Software Inc, San Diego, CA, U.S.A.). Every experiment showed in this study was performed in HPAECs from almost three different patients in n = 3-4 experiments per patient. Significance was accepted when $P < 0.05$.

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Results

CSE-induced up-regulation of ET_B in HPAECs is partially suppressed by bosentan.

CSE dose-dependently increased ET_B mRNA and protein expression in HPAECs after 24 h of incubation (Figure 1A). Interestingly, CSE-induced ET_B over-expression was inhibited by bosentan in a concentration-dependent manner (10nM-10μM; Figure 1B), reaching statistical significance at 100nM (Figure 1B; P<0.05 *vs.* CSE 10%). In this sense, the selective ET_B antagonist BQ788 also reduced, in a concentration-dependent manner, the CSE-induced ET_B over-expression (10nM-10μM; Figure 1C), suggesting a feed forward mechanism mediated via ET receptors. These results were also confirmed

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by immune-fluorescence staining with ET_B antibody (Figure 1D). Pharmacologic treatment with bosentan or BQ788 alone did not show any effect on basal ET_B expression (data not shown). To further study the mechanistic effect of the CSE-induced ET_B over-expression, the amount of ET in cell culture supernatants were measured. At this respect, CSE dose-dependently released ET from HPAECs, reaching statistical significance at 5-10% concentrations (Figure 2A; $P<0.05$ *vs*. basal conditions). Furthermore, the addition of mAb-ET-1 10µg/ml, significantly reduced the CSE-induced ET_B over-expression suggesting that the ET-1 released by CSE is involved in this process (Figure 2B; $P<0.05$ *vs*. CSE 10%). In this sense, ET-1 stimulation dose-dependently (100pM-100nM) increased ET_B expression. These results demonstrate that ET-1 is one of the downstream effectors of CSE causing ET_B over-expression.

The CSE-induced ET_B over-expression is partially mediated by the activation of ET_B and the downstream pathway Rho kinase and ROS production.

Since it is known that CS promotes RhoA and MLCK activation and ROS production, we studied the effect of these downstream pathways on the CSE-induced ET_B over-expression. As it was observed for bosentan (10 µM), the Rho kinase inhibitor, Y27632 (10 µM) and the antioxidant NAC (1mM) effectively reversed the CSE-induced mRNA and protein ET_B over-expression (Figure 3A). In contrast, the MLCK inhibitor ML-7 did not show any significant effect (Figure 3A). When ET-1 (10nM) was employed as inductor of ET_B expression, bosentan (10 µM), BQ788 (1µM), Y27632 (10 µM) and the antioxidant NAC (1mM) partially suppressed the ET_B over-expression (Figure 3B) unlike ML-7, reinforcing the role of ET-1 on CSE-induced ET_B up-regulation. In other experiments we explored the possible additive effects of bosentan, Y27632 and NAC. To this respect, we selected concentrations of bosentan (100nM), Y27632 (1µM) and

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NAC (100µM) which reached n 50% inhibition of the maximal effect of CSE (Figure 3C). Thus, the combination of these three inhibitors showed an additive effect on reduction of the CSE-induced ET_B up-regulation (Figure 3C; $P<0.05$ *vs*. bosentan, Y27632 and NAC groups).

To further investigate the role of RhoA on CSE-induced ET_B up-regulation, we selectivity silenced RhoA RNA. siRNA targeted at RhoA produced a marked inhibition in mRNA and protein expression following 48 h exposure to 50 nM siRNA-RhoA (Figure 3D; P<0.05 vs. siRNA-control). siRNA targeted at RhoA prevented the CSE-induced ET_B over-expression confirming those results observed for Y27632 (10 µM) (Figure 3E; $P<0.05$ vs. siRNA-control plus CSE 10%).

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HPAECs exposed to CSE increase sensitivity to ET-1-induced $[Ca^{2+}]_i$.

The $[Ca^{2+}]_i$ mediates several cellular process such as endothelial cell contraction and permeabilization, proliferation and inflammation among others (Garcia *et al.*, 1993). Since CSE increases ET_B expression, we investigated the effect of acute ET-1 on $[Ca^{2+}]_i$ in HPAECs pre-treated for 24 h with or without CSE in presence or absence of bosentan (10µM) or BQ788 (1µM). Preliminary experiments showed that ET-1 increased $[Ca^{2+}]_i$ on HPAECs in a dose-dependent manner with a potency of ~23nM ($-logEC_{50}=7.62\pm0.06$, data not shown). Therefore, ET-1 10nM was employed in subsequent experiments. HPAECs stimulated with CSE 10% for 24 h showed a $[Ca^{2+}]_i$ baseline of 120.2±4.6 nM significantly higher than that observed for unstimulated cells ($[Ca^{2+}]_i$ baseline of 93±3.4nM; $P<0.05$). Furthermore, ET-1 (10nM) stimulation gave rise to a significant increment of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) of 214.5±12.6 nM in CSE treated cells *vs*. untreated cell ($\Delta[Ca^{2+}]_i$ = 142.8±6.3 nM; $P<0.05$) (Figure 4A, 4B and Table 1). In other experiments, bosentan (0.1-10µM) or BQ788 (1µM) were added 1 h before

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CSE 10% an incubated together for 24 h. In these conditions, bosentan (1- 10μM) and BQ788 (1μM) significantly reduced the acute effect of ET-1 10nM on $[Ca^{2+}]_i$ (Figure 4A, 4B and Table 1; $P<0.05$).

ET-1 increases cell contraction and F/G actin imbalance in HPAECs pretreated with CSE.

To elucidate whether the increased sensitivity to ET-1-induced $[Ca^{2+}]_i$ may be translated into cell contraction, we employed traction microscopy technique. ET-1 (10nM) produced a fast and sustained increase in cell contraction that was significantly higher in those cells exposed to CSE 10% for 24 h *vs*. unexposed cells (Figure 5A; $P<0.05$). Furthermore, those cells pre-treated with CSE in the presence of bosentan (10 μM), BQ788 (1μM), Y27632 (10 μM) or ML-7 nearly suppressed cell contraction to control levels (Figure 5A).

Cytoskeleton rearrangement is a direct marker of cell contraction, and polymerization of soluble G-actin to F-actin fibers are part of these process (Gavara *et al.*, 2006). The F/G-actin fluorescence ratio value of HPAECs exposed to CSE 10% for 24 h did not show significant difference to those cells unexposed to CSE (Figure 5B). In contrast, the CSE-treated cells showed an increased F/G-actin ratio in response to acute ET-1 10nM (30 min) (F/G-actin ratio 2.95 ± 0.3 in cells exposed to CSE *vs*. 1.6±0.2 in cells unexposed; Figure 5C; $P<0.05$). The addition of bosentan (0.1-10μM), BQ788 (1μM), Y27632 (10μM) or ML-7 (10μM) 1 h before CSE 10%, significantly reduced the increase of F/G-actin ratio induced by ET-1 (Figure 5C; $P<0.05$ *vs*. CSE).

Because RhoA kinase inhibitor and ML-7 inhibitor attenuate the effect of CSE on ET-1-induced cell contraction we explore the activation of RhoA and the phosphorylation of MLC in those cells exposed to CSE. In this regard, the basal RhoA activity and MLC

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phosphorylation did not differ from CSE exposed cell to unexposed cell (measure after 24 h of exposure; Figure 6A and 6B). On the other hand, acute ET-1 (10nM, 30 min) increased RhoA activity in those cells exposed to CSE over unexposed cells (Figure 6C; $P<0.05$). Furthermore, when bosentan (1µM and 10 µM), BQ788 (1µM) and Y27632 (10 µM) where added before CSE (for 24 h) the ET-1-induced RhoA activity was partially abrogated (Figure 6C; $P<0.05$ *vs*. CSE). On the other hand CSE 10% increased the ET-1-induced MLC phosphorylation which was attenuated by bosentan (10 µM), BQ788 (1µM), Y27632 (10 µM) and ML-7 (10 µ M) (Figure 6D).

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ET-1 reduces NO release and increases TxA_2 in HPAECs pretreated with CSE.

It is well established that CS reduces NO and increases the release of TxA_2, however no data is reported about the influence of CS on ET-1-induced NO and TxA_2 release. Since CSE increases ET_B expression, the ET-1 effects on NO and TxA_2 release could be different. In this regard, HPAECs exposed to CSE for 24 h were stimulated with acute ET-1 10nM for 1 h. Then, the stable derivatives of NO and TxA_2, nitrites and TxB_2 were measured in the cell culture supernatant. We observed that CSE exposure reduced the ET-1-induced nitrite release by n 50% respect unexposed cells (Figure 7A, $P<0.05$). Furthermore, pharmacological addition of bosentan (1µM and 10 µM), BQ788 (1µM), Y27632 (10 µM) as well as the antioxidant NAC (1mM) significantly improved the nitrite release by ET-1 (Figure 5A; $P<0.05$ *vs*. CSE). In contrast, ML-7 (10 µM) failed to modify NO release. On the other hand, HPAECs treated with CSE increased the ET-1-induced TxB_2 release by n 2 fold (Figure 5B, $P<0.05$ *vs*. unexposed cells to CSE). Bosentan (1µM and 10 µM), BQ788 (1µM), Y27632 (10 µM) and NAC (1mM) significantly reduced the TxB_2 release by ET-1 (Figure 5A; $P<0.05$ *vs*. CSE), while ML-7 did not show any effect.

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ET-1 increases intracellular ROS production in HPAECs pretreated with CSE.

ROS are the main responsible of the CS intracellular actions in endothelial cells. Thus, first we evaluated the CSE-induced ROS production. In this sense, CSE increased intracellular ROS in time and concentration dependent manner reaching a peak value after 10 min of stimulation (Figure 8A) followed by a subsequent decline that reached a plateau above baseline after ~30 min (Figure 8A). The antioxidant NAC (1mM) completely prevented ROS generation (Figure 8A). Secondly, we studied the role of CSE on the ET-1-induced ROS production. Since ET-1 10nM reached sub maximal ROS production after 10 min of stimulation (Figure 8B), we selected these experimental conditions in further experiments. HPAECs stimulated with CSE 10% for 24 h showed an increase of ROS production in response to acute ET-1 which was significantly higher than in HPAECs unexposed to CSE (Figure 6C; $P<0.05$ *vs*. unexposed cells). When bosentan (1μM and 10 μM), BQ788 (1μM), Y27632 (10 μM) and NAC (1mM) were added to HPAECs before CSE, the effect of ET-1 on ROS production was suppressed (Figure 8C; $P<0.05$ *vs*. CSE).

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Discussion

This study was designed to evaluate the effect of CS on ET_B expression in HPAECs and its functional consequences in a context of ET-1 exposure *in vitro*. We found for the first time that HPAECs stimulated with CSE increase ET_B expression *via* a feed forward mechanism involving ET release from CSE-exposed cells. In this regard, bosentan as well as the selective ET_B inhibitor BQ788 prevented the CSE-induced ET_B up-regulation. Furthermore, the ET_B over-expression in a context of CSE exposure sensitized HPAECs to ET-1 in those aspects involving endothelial dysfunction such as cell contraction, NO reduction, TxA_2 increase as well as an increase of ROS production.

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These results introduce a novel concept by which in an inflammatory context such as CS, the up-regulation of ET_B potentiates endothelial dysfunction induced by ET-1. Nowadays the studies focused in ET_B function on endothelial cells indicate a protective role, since its stimulation releases vasodilators and antiangiogenic factors such as NO and PGI_2. It has been shown that endothelial ET_B knockout mice develops severe right ventricular pressure during hypoxia (Kelland *et al.,* 2010), and that endothelial ET_B remove ET-1 from systemic circulation to lung tissue. In contrast to ET_A, the ET_B is an inducible protein in both smooth muscle cells and endothelial cells (Galie *et al.*, 2004; Yeligar *et al.*, 2009). In this regard, in thromboembolic PH and in PH derived from systemic sclerosis as well as in inflammatory conditions, ET_B expression is up-regulated mainly in pulmonary arterial smooth muscle cells where ET_B mediates contraction and smooth muscle cell proliferation (Bauer *et al.*, 2002; Frommer *et al.*, 2008) contributing to pulmonary remodelling. Furthermore, CS also up-regulates ET_B expression on pulmonary artery smooth muscle cells inducing pulmonary artery contraction (Xu *et al.*, 2008). However, the role of CS on ET_B expression and its functional consequences in endothelial function remains to be elucidated. In this work we focused on the effect of CS as an inflammatory condition which may represent the initial endothelial dysfunction that could develop PH in COPD patients. We detected that CSE induces ET_B over-expression in HPAECs and that it may be prevented by the ET_B/ET_A antagonist bosentan, the selective ET_B antagonist BQ788 as well as by the inhibition of Rho kinase and ROS. It is known that CS induces RhoA activation and ROS generation in endothelial cells promoting endothelial dysfunction (Milara *et al.,* 2010; Richens *et al.*, 2009; Sugimoto *et al.*, 2007), and that ROS generation may up-regulate ET_B expression (Yeligar *et al.*, 2009). Since CSE induces ET release (Wright *et al.*, 2006), and the activation of ET_B induces ROS production (Dong *et al.*, 2005), seems

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reasonable to think that bosentan and BQ788 may inhibit the CSE-induced ET_B over-expression. In this regard, we detected an increased ET concentration following CSE exposure. Furthermore, the addition of mAb-ET-1 partially suppressed the CSE-induced ET_B over-expression. This finding was reinforced by the direct ET_B up-regulation induced by ET-1, which suggests that the CSE-induced ET_B over-expression is mediated in part by a feed forward mechanism.

Although the ET_B expression in endothelial cells is related with beneficial effects, its over-expression in an inflammatory context has been scarcely examined. Thus, we investigated the functional role of the CSE-induced ET_B expression in HPAECs.

Endothelial permeability may be an important component of the pathogenesis of PH. Thus, an augmented endothelial permeability may increase inflammatory cell infiltrating the wall of pulmonary arteries, and enlarge the intimal layer promoting pulmonary remodelling. In this sense, it has been hypothesized that the development of structural and functional abnormalities of pulmonary arteries in COPD is related to inflammatory cells in the vascular wall (Wright *et al.*, 2005).

The GTPasa RhoA and the MLCK play a key role on cell contraction and permeability (Oka *et al.*, 2008). It is know that ET_B stimulation induces RhoA activation and intracellular Ca^{2+} release, and that both mediate MLC phosphorylation which increases formation of F-actin stress fibers, cell contraction and permeability (Morrell *et al.*, 2009; Oka *et al.*, 2008). Consistent with these findings, *in vivo* studies have shown that selective ET_B activation increases albumin extravasation in guinea pig lungs (Filep *et al.*, 1995). In this work, we observed that HPAECs exposed to CSE, which is in agreement with ET_B over-expression, sensitizes cells to acute ET-1. Thus, CSE-exposed cells showed an increased response to acute ET-1 in those aspects related with cell permeability such as the increase of $[Ca^{2+}]_i$, RhoA activation, MLC-phosphorylation, F-

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actin polymerization and cell contraction. Since pharmacological pre-treatments with bosentan, BQ788 and Y27632 reduced this process and prevent the ET_B over-expression, we may corroborate that ET-1-induced endothelial permeability in cells exposed to CSE is mediated in part by the induction of ET_B expression.

Next, we focused on the effect of chronic CSE in ET-1-induced NO/ TxA_2 release. The balance of the vasodilator/vasoconstrictor cell release is a key point related to endothelial cell function. In physiological conditions HPAECs mainly release the vasodilators and antiangiogenics NO and PGI_2. However, in a cell injury context, this balance may be altered promoting the release of vasoconstrictors on the vasodilators (Christman *et al.*, 1992). There is strong evidence that part of the ET-1-induced vasoconstriction is modulated by TxA_2 production, which is mediated by the ET_B and the vascular endothelium (Curzen *et al.*, 1995; D'Orleans-Juste *et al.*, 1994). The endothelial ET_B may thus have a dual role, modulating both vasoconstriction and vasodilation, and these effects could, in turn, be modulated by pathological conditions affecting endothelial function itself. In the present work we observed that HPAECs treated with CSE sensitized cells to produce less NO and more TxA_2 in response to acute ET-1. Furthermore, this effect was attenuated by bosentan, BQ788, Y27632 and NAC, which suggest that ET_B, Rho kinase and ROS are implicated in this process.

It is well established that CS inhibits the eNOS activity and reduces NO bioavailability through the interaction of NO with ROS to form peroxynitrite. Furthermore, as we have commented before, CSE induces RhoA and the downstream Rho kinase activation that can directly phosphorylate eNOS at Thr459 to suppress NO production in endothelium (Sugimoto *et al.*, 2007). Intracellular ROS produced by CSE and ET-1 could explain in part the imbalance between the vasodilators/vasoconstrictors release. Thus, ET-1-induced ROS production was highest in HPAECs exposed to CSE suggesting that ET_B

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over-expression increases ROS production. Furthermore, bosentan, BQ788, Y27632 and NAC completely suppressed the effect of CSE on ET-1-induced ROS. Since, these drugs also prevented the ET_B over-expression secondary to CSE, we may postulate that the ET-1-induced ROS in CSE-exposed cells is mediated, at least in part, by ET_B over-expression.

In summary, we would like to suggest the following model by which CSE induces ET_B over-expression by a feed forward mechanism mediated in part by the ET cell releases, promoting HPAEC dysfunction which is partially attenuated by ET_B blockage, Rho kinase and ROS inhibition. These results may provide in vitro evidence of the use of bosentan in CS-related endothelial dysfunction.

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Bibliography

Barbera, JA, Blanco, I (2009) Pulmonary hypertension in patients with chronic obstructive pulmonary disease: advances in pathophysiology and management. *Drugs* **69**(9)**:** 1153-1171.

Barua, RS, Ambrose, JA, Srivastava, S, DeVoe, MC, Eales-Reynolds, LJ (2003) Reactive oxygen species are involved in smoking-induced dysfunction of nitric oxide biosynthesis and upregulation of endothelial nitric oxide synthase: an in vitro demonstration in human coronary artery endothelial cells. *Circulation* **107**(18)**:** 2342-2347.

Bauer, M, Wilkens, H, Langer, F, Schneider, SO, Lausberg, H, Schafers, HJ (2002) Selective upregulation of endothelin B receptor gene expression in severe pulmonary hypertension. *Circulation* **105**(9)**:** 1034-1036.

Bernhard, D, Csordas, A, Henderson, B, Rossmann, A, Kind, M, Wick, G (2005) Cigarette smoke metal-catalyzed protein oxidation leads to vascular endothelial cell contraction by depolymerization of microtubules. *FASEB J* **19**(9)**:** 1096-1107.

Cacoub, P, Dorent, R, Nataf, P, Carayon, A, Riquet, M, Noe, E, Piette, JC, Godeau, P, Gandjbakhch, I (1997) Endothelin-1 in the lungs of patients with pulmonary hypertension. *Cardiovasc Res* **33**(1)**:** 196-200.

Carratu, P, Scoditti, C, Maniscalco, M, Seccia, TM, Di Gioia, G, Gadaleta, F, Cardone, RA, Dragonieri, S, Pierucci, P, Spanevello, A, Resta, O (2008) Exhaled and arterial

For Peer Review

levels of endothelin-1 are increased and correlate with pulmonary systolic pressure in COPD with pulmonary hypertension. *BMC Pulm Med* **8:** 20.

Cortijo, J, Milara, J, Mata, M, Donet, E, Gavara, N, Peel, SE, Hall, IP, Morcillo, EJ (2010) Nickel induces intracellular calcium mobilization and pathophysiological responses in human cultured airway epithelial cells. *Chem Biol Interact* **183**(1)**:** 25-33.

Cramer, LP, Briggs, LJ, Dawe, HR (2002) Use of fluorescently labelled deoxyribonuclease I to spatially measure G-actin levels in migrating and non-migrating cells. *Cell Motil Cytoskeleton* **51**(1)**:** 27-38.

Curzen, NP, Griffiths, MJ, Evans, TW (1995) Contraction to endothelin-1 in pulmonary arteries from endotoxin-treated rats is modulated by endothelium. *Am J Physiol* **268**(6 Pt 2)**:** H2260-2266.

Christman, BW, McPherson, CD, Newman, JH, King, GA, Bernard, GR, Groves, BM, Loyd, JE (1992) An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N Engl J Med* **327**(2)**:** 70-75.

D'Orleans-Juste, P, Claing, A, Telemaque, S, Maurice, MC, Yano, M, Gratton, JP (1994) Block of endothelin-1-induced release of thromboxane A2 from the guinea pig lung and nitric oxide from the rabbit kidney by a selective ETB receptor antagonist, BQ-788. *Br J Pharmacol* **113**(4)**:** 1257-1262.

For Peer Review

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Dalli, E, Milara, J, Cortijo, J, Morcillo, EJ, Cosin-Sales, J, Sotillo, JF (2008) Hawthorn extract inhibits human isolated neutrophil functions. *Pharmacol Res* **57**(6)**:** 445-450.

Didier, N, Romero, IA, Creminon, C, Wijkhuisen, A, Grassi, J, Mabondzo, A (2003) Secretion of interleukin-1beta by astrocytes mediates endothelin-1 and tumour necrosis factor-alpha effects on human brain microvascular endothelial cell permeability. *J Neurochem* **86**(1)**:** 246-254.

Dinh-Xuan, AT, Higenbottam, TW, Clelland, CA, Pepke-Zaba, J, Cremona, G, Butt, AY, Large, SR, Wells, FC, Wallwork, J (1991) Impairment of endothelium-dependent pulmonary-artery relaxation in chronic obstructive lung disease. *N Engl J Med* **324**(22)**:** 1539-1547.

Dong, F, Zhang, X, Wold, LE, Ren, Q, Zhang, Z, Ren, J (2005) Endothelin-1 enhances oxidative stress, cell proliferation and reduces apoptosis in human umbilical vein endothelial cells: role of ETB receptor, NADPH oxidase and caveolin-1. *Br J Pharmacol* **145**(3)**:** 323-333.

Dupuis, J, Goresky, CA, Fournier, A (1996) Pulmonary clearance of circulating endothelin-1 in dogs in vivo: exclusive role of ETB receptors. *J Appl Physiol* **81**(4)**:** 1510-1515.

Eddahibi, S, Chaouat, A, Tu, L, Chouaid, C, Weitzenblum, E, Housset, B, Maitre, B, Adnot, S (2006) Interleukin-6 gene polymorphism confers susceptibility to pulmonary

For Peer Review

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hypertension in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* **3**(6)**:** 475-476.

Filep, JG, Fournier, A, Foldes-Filep, E (1995) Acute pro-inflammatory actions of endothelin-1 in the guinea-pig lung: involvement of ETA and ETB receptors. *Br J Pharmacol* **115**(2)**:** 227-236.

Frommer, KW, Muller-Ladner, U (2008) Expression and function of ETA and ETB receptors in SSc. *Rheumatology (Oxford)* **47 Suppl 5:** v27-28.

Galie, N, Manes, A, Branzi, A (2004) The endothelin system in pulmonary arterial hypertension. *Cardiovasc Res* **61**(2)**:** 227-237.

Garcia, JG, Patterson, C, Bahler, C, Aschner, J, Hart, CM, English, D (1993) Thrombin receptor activating peptides induce Ca2+ mobilization, barrier dysfunction, prostaglandin synthesis, and platelet-derived growth factor mRNA expression in cultured endothelium. *J Cell Physiol* **156**(3)**:** 541-549.

Gavara, N, Sunyer, R, Roca-Cusachs, P, Farre, R, Rotger, M, Navajas, D (2006) Thrombin-induced contraction in alveolar epithelial cells probed by traction microscopy. *J Appl Physiol* **101**(2)**:** 512-520.

Granstrom, BW, Xu, CB, Nilsson, E, Vikman, P, Edvinsson, L (2006) Smoking particles enhance endothelin A and endothelin B receptor-mediated contractions by enhancing translation in rat bronchi. *BMC Pulm Med* **6:** 6.

For Peer Review

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Grynkiewicz, G, Poenie, M, Tsien, RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* **260**(6)**:** 3440-3450.

Hewett, PW, Murray, JC (1993) Immunomagnetic purification of human microvessel endothelial cells using Dynabeads coated with monoclonal antibodies to PECAM-1. *Eur J Cell Biol* **62**(2)**:** 451-454.

Holm, P, Franco-Cereceda, A (1996) Tissue concentrations of endothelins and functional effects of endothelin-receptor activation in human arteries and veins. *J Thorac Cardiovasc Surg* **112**(2)**:** 264-272.

Joppa, P, Petrasova, D, Stancak, B, Tkacova, R (2006) Systemic inflammation in patients with COPD and pulmonary hypertension. *Chest* **130**(2)**:** 326-333.

Kakoki, M, Hirata, Y, Hayakawa, H, Tojo, A, Nagata, D, Suzuki, E, Kimura, K, Goto, A, Kikuchi, K, Nagano, T, Omata, M (1999) Effects of hypertension, diabetes mellitus, and hypercholesterolemia on endothelin type B receptor-mediated nitric oxide release from rat kidney. *Circulation* **99**(9)**:** 1242-1248.

Kelland, NF, Bagnall, AJ, Morecroft, I, Gulliver-Sloan, FH, Dempsie, Y, Nilsen, M, Yanagisawa, M, Maclean, MR, Kotelevtsev, YV, Webb, DJ Endothelial ET(B) (2010) limits vascular remodelling and development of pulmonary hypertension during hypoxia. *J Vasc Res* **47**(1)**:** 16-22.

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Migneault, A, Sauvageau, S, Villeneuve, L, Thorin, E, Fournier, A, Leblanc, N, Dupuis, J (2005) Chronically elevated endothelin levels reduce pulmonary vascular reactivity to nitric oxide. *Am J Respir Crit Care Med* **171**(5)**:** 506-513.

Milara, J, Juan, G, Ortiz, JL, Guijarro, R, Losada, M, Morcillo, EJ, Cortijo, J (2010) Cigarette smoke-induced pulmonary endothelial dysfunction is partially suppressed by sildenafil. *Eur J Pharm Sci*.

Moller, S, Uddman, R, Granstrom, B, Edvinsson, L (1999) Altered ratio of endothelin ET(A)- and ET(B) receptor mRNA in bronchial biopsies from patients with asthma and chronic airway obstruction. *Eur J Pharmacol* **365**(1)**:** R1-3.

Morrell, NW, Adnot, S, Archer, SL, Dupuis, J, Jones, PL, MacLean, MR, McMurtry, IF, Stenmark, KR, Thistlethwaite, PA, Weissmann, N, Yuan, JX, Weir, EK (2009) Cellular and molecular basis of pulmonary arterial hypertension. *J Am Coll Cardiol* **54**(1 Suppl)**:** S20-31.

Nana-Sinkam, SP, Lee, JD, Sotto-Santiago, S, Stearman, RS, Keith, RL, Choudhury, Q, Cool, C, Parr, J, Moore, MD, Bull, TM, Voelkel, NF, Geraci, MW (2007) Prostacyclin prevents pulmonary endothelial cell apoptosis induced by cigarette smoke. *Am J Respir Crit Care Med* **175**(7)**:** 676-685.

Oka, M, Fagan, KA, Jones, PL, McMurtry, IF (2008) Therapeutic potential of RhoA/Rho kinase inhibitors in pulmonary hypertension. *Br J Pharmacol* **155**(4)**:** 444-454.

For Peer Review

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Orosz, Z, Csiszar, A, Labinskyy, N, Smith, K, Kaminski, PM, Ferdinandy, P, Wolin, MS, Rivera, A, Ungvari, Z (2007) Cigarette smoke-induced proinflammatory alterations in the endothelial phenotype: role of NAD(P)H oxidase activation. *Am J Physiol Heart Circ Physiol* **292**(1)**:** H130-139.

Ortiz, JL, Milara, J, Juan, G, Montesinos, JL, Mata, M, Ramon, M, Morcillo, E, Cortijo, J (2009) Direct effect of cigarette smoke on human pulmonary artery tension. *Pulm Pharmacol Ther*.

Peinado, VI, Barbera, JA, Abate, P, Ramirez, J, Roca, J, Santos, S, Rodriguez-Roisin, R (1999) Inflammatory reaction in pulmonary muscular arteries of patients with mild chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **159**(5 Pt 1)**:** 1605-1611.

Peinado, VI, Barbera, JA, Ramirez, J, Gomez, FP, Roca, J, Jover, L, Gimferrer, JM, Rodriguez-Roisin, R (1998) Endothelial dysfunction in pulmonary arteries of patients with mild COPD. *Am J Physiol* **274**(6 Pt 1)**:** L908-913.

Richens, TR, Linderman, DJ, Horstmann, SA, Lambert, C, Xiao, YQ, Keith, RL, Boe, DM, Morimoto, K, Bowler, RP, Day, BJ, Janssen, WJ, Henson, PM, Vandivier, RW (2009) Cigarette smoke impairs clearance of apoptotic cells through oxidant-dependent activation of RhoA. *Am J Respir Crit Care Med* **179**(11)**:** 1011-1021.

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Santos, S, Peinado, VI, Ramirez, J, Melgosa, T, Roca, J, Rodriguez-Roisin, R, Barbera, JA (2002) Characterization of pulmonary vascular remodelling in smokers and patients with mild COPD. *Eur Respir J* **19**(4)**:** 632-638.

Stenmark, KR, Fagan, KA, Frid, MG (2006) Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. *Circ Res* **99**(7)**:** 675-691.

Su, Y, Han, W, Giraldo, C, De Li, Y, Block, ER (1998) Effect of cigarette smoke extract on nitric oxide synthase in pulmonary artery endothelial cells. *Am J Respir Cell Mol Biol* **19**(5)**:** 819-825.

Sugimoto, M, Nakayama, M, Goto, TM, Amano, M, Komori, K, Kaibuchi, K (2007) Rho-kinase phosphorylates eNOS at threonine 495 in endothelial cells. *Biochem Biophys Res Commun* **361**(2)**:** 462-467.

Voelkel, NF, Cool, CD (2003) Pulmonary vascular involvement in chronic obstructive pulmonary disease. *Eur Respir J Suppl* **46:** 28s-32s.

Wright, JL, Levy, RD, Churg, A (2005) Pulmonary hypertension in chronic obstructive pulmonary disease: current theories of pathogenesis and their implications for treatment. *Thorax* **60**(7)**:** 605-609.

Wright, JL, Tai, H, Churg, A (2004) Cigarette smoke induces persisting increases of vasoactive mediators in pulmonary arteries. *Am J Respir Cell Mol Biol* **31**(5)**:** 501-509.

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Wright, JL, Tai, H, Churg, A (2006) Vasoactive mediators and pulmonary hypertension after cigarette smoke exposure in the guinea pig. *J Appl Physiol* **100**(2)**:** 672-678.

Xu, CB, Zheng, JP, Zhang, W, Zhang, Y, Edvinsson, L (2008) Lipid-soluble smoke particles upregulate vascular smooth muscle ETB receptors via activation of mitogen-activating protein kinases and NF-kappaB pathways. *Toxicol Sci* **106**(2)**:** 546-555.

Yeligar, S, Tsukamoto, H, Kalra, VK (2009) Ethanol-induced expression of ET-1 and ET-BR in liver sinusoidal endothelial cells and human endothelial cells involves hypoxia-inducible factor-1alpha and microrNA-199. *J Immunol* **183**(8)**:** 5232-5243.

For Peer Review

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Legend to figures

Figure 1

CSE-induced ET_B expression in HPAECs is attenuated by bosentan

HPAECs were incubated with different CSE concentrations for 24 h. A) Then, mRNA and protein for ET_B were quantified by real time RT-PCR and western blot respectively. B, C) HPAECs were incubated with bosentan (10nM-10µM) or BQ788 (10nM-10µM) 1 h before CSE addition. Bosentan and BQ788 dose-dependently attenuate ET_B mRNA and protein expression measured by real time RT-PCR, western blot. D) Immunofluorescence for ET_B shows an increment of ET_B expression (red color) in cells exposed to CSE which was prevented by bosentan (DAPI-blue colour represents nucleus). Scale bar: 10µm. Results are the mean ±SEM of 4 different experiments from 3 different patients per condition. **P*<0.05 *vs*. basal conditions; #*P*<0.05 *vs*. CSE 10%.

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Figure 2

CSE-induced ET_B over-expression is partially mediated by supernatant ET levels

A) CSE dose-dependently releases ET to cell culture supernatant after 24 h of CSE-exposure. B) The CSE-induced ET_B over-expression was partially inhibited by mAb-ET1 (10µg/ml) and not by the isotype IgG1 control (10µg/ml). C) Furthermore, ET-1 exposure for 24 h, dose-dependently increased ET_B mRNA and protein expression. Results are the mean ±SEM of 4 different experiments from 3 different patients per condition. **P*<0.05 *vs*. basal conditions; #*P*<0.05 *vs*. CSE 10%.

Figure 3

CSE-induced ET_B over-expression is attenuated by the inhibition of ET_B, Rho kinase and ROS

A) HPAECs were incubated with bosentan, Y27632, NAC or ML-7 1 h before the addition of CSE 10%. After 24 h of incubation, ET_B mRNA and protein were quantified by real time RT-PCR and western blot respectively. B) HPAECs were incubated with bosentan, BQ788, Y27632, NAC or ML-7 1 h before the addition of ET-1 (10nM). After 24 h of incubation, ET_B mRNA and protein were quantified. C) Additive effects of Bosentan, Y27632 and NAC at n 50% effective concentrations showed additive effects on the inhibition of CSE-induced ET_B-up-regulation. D) Specific siRNA targeted to RhoA effectively suppressed RhoA mRNA and protein expression in HPAECs. E) siRNA-RhoA suppressed CSE-induced ET_B increase in HPAECs (presented as mRNA and protein expression) respect cells transfected with a negative control siRNA. Results are the mean ±SEM of 3 different experiments from 3 different patients per condition. **P*<0.05 *vs*. control; #*P*<0.05 *vs*. CSE 10%.

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Figure 4

HPAECs exposed to CSE for 24 h increase the acute ET-induced $[Ca^{2+}]_i$

A) HPAECs were exposed to vehicle, bosentan or BQ778 for 24 h. Then cells were washed three times with PBS and incubated with fura 2AM. The increase of $[Ca^{2+}]_i$ following acute ET-1 (10nM) was the same for each condition. B) HPAECs were exposed to CSE, in presence or absence of bosentan or BQ778 for 24 h. Then cells were washed three times with PBS and incubated with fura 2AM. The increase of $[Ca^{2+}]_i$ following acute ET-1 (10nM) was higher in those cells exposed to CSE 10%. Results are the mean ±SEM of $[Ca^{2+}]_i$ nM in 12 cells per experiment in a total of 4 experiments per condition.**P*<0.05 *vs*. control; #*P*<0.05 *vs*. CSE 10%.

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Figure 5

ET-1 increases cell contraction and F/G actin imbalance in HPAECs pretreated with CSE

A) Disks with cultured HPAECs were incubated for 24 h in absence (control) or presence of CSE 10% alone or in combination (1 h before CSE) with bosentan, BQ788, ML-7 or Y27632. Then, gel disk with cultured HPAECs was placed in the microscope and cells imaged with bright-field illumination. Graphic shows the time course of contractile response of HPAECs challenged with ET-1 (10nM). Values are expressed as total force exerted by the cell on the substrate. B) HPAECs were treated with or without CSE for 24 h. Then, cells were fixed with 3.7% formaldehyde and phalloidin-tetramethylrhodamine isothiocyanate and Alexa Fluor 488 DNase I conjugate were added to mark F and G actin respectively. DAPI was added to mark cell nucleus. Images are representative of the F-actin (red colour) and G-actin (green color) staining, and graphic shows the basal F/G actin fluorescence ratio after these experimental conditions. C) In other experiments, bosentan, BQ788, Y27632 or ML-7 were added to cell culture 1 h before CSE. After 24 h of incubation cells were washed three times with PBS and stimulated with ET-1 10nM for 30 min. Then, cells were treated to mark F and G actin. DAPI was added to mark cell nucleus (blue color). HPAECs images show representative experiments under these experimental conditions. Scale bar: 10µm. Graphic represents the mean ±SEM of the fluorescence intensities of F-actin (red) versus G-actin (green) in a total of 3 different experiments from 3 different patients per condition. *$P<0.05$ *vs*. control; #$P<0.05$ *vs*. CSE.

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Figure 6

HPAECs exposed to CSE sensitizes cell to ET-1-induced RhoA activation and MLC-phosphorylation.

A) RhoA activation was measured in HPAECs treated or not with CSE 10% for 24 h by commercial colorimetric kit. B) After 24 h of CSE exposure MLC-phosphorylation was measured by western blot. C, D) HPAECs were incubated with CSE for 24 h. After CSE treatment cells were washed three times with PBS and stimulated with ET-1 10nM for 30 min, and RhoA activity (C) and MLC phosphorylation (D) were measured. Cells exposed to CSE showed an increase of RhoA activity and p-MLC compared to cells unexposed. Furthermore, the incubation with bosentan, BQ788, Y27632 and ML-7 (only in p-MLC experiments) 1 h before of CSE addition, attenuate the ET-1-induced RhoA activation and MLC phosphorylation. Results are the mean ±SEM of the absorbance corresponding to active RhoA and representative western blot for p-MLC in a total of 3 different experiments from 3 different patients per condition. *P<0.05 *vs*. control; #P<0.05 *vs*. CSE 10%.

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Figure 7

HPAECs exposed to CSE increase the imbalance between NO and TxA_2 release in response to acute ET-1.

HPAECs were cultured in 6-well flasks until ∩ 95% confluent. Then, cells were incubated with or without CSE 10% for 24 h. In parallel experiments, bosentan, BQ788, Y27632, NAC or ML-7 were added to cell culture 1 h before CSE. After 24 h of incubation, cells were washed three times with PBS and stimulated with ET-1 for 30 min. CSE-treated cells significantly decrease NO release (measured as nitrites) (A) and

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increase TxA_2 release (measured as TxB_2) (B) in response to acute ET-1. Furthermore, cell incubation with bosentan, BQ788, Y27632 and NAC attenuated the effect of CSE on ET-1-induced NO decrease and TxA_2 increase. Results are the mean ±SEM of a total of 3 different experiments from 3 different patients per condition. *P<0.05 *vs*. control; #P<0.05 *vs*. CSE 10%.

Figure 8

ET-1 increases ROS production in HPAECs treated with CSE

A, B) Confluent HPAEC were cultured in black walled clear bottom 96 well plates and loaded with DCFDA for 30 min. Then, cells were exposed to (A) CSE (2.5%-10%) or (B) ET-1 (0.1-100nM) and DCF fluorescence was monitored each 5 min during 45 min. D) In other experiments, cells were incubated with or without CSE 10% in presence or absence of bosentan, BQ788, Y27632, NAC or ML-7 for 24 h. Then, cells were washed three times with PBS and loaded with DCFDA for 30 min. Thus, cells were stimulated with ET-1 (10nM) and DCF fluorescence was measured after 10 min of stimulation. Results are the mean ±SEM of a total of 6 different experiments from 3 different patients per condition. *P<0.05 *vs*. control; #P<0.05 *vs*. CSE 10%.

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Statement of conflicts of interest

This work has been partially supported by Actelion Pharmaceuticals Ltd, Switzerland which provided bosentan.

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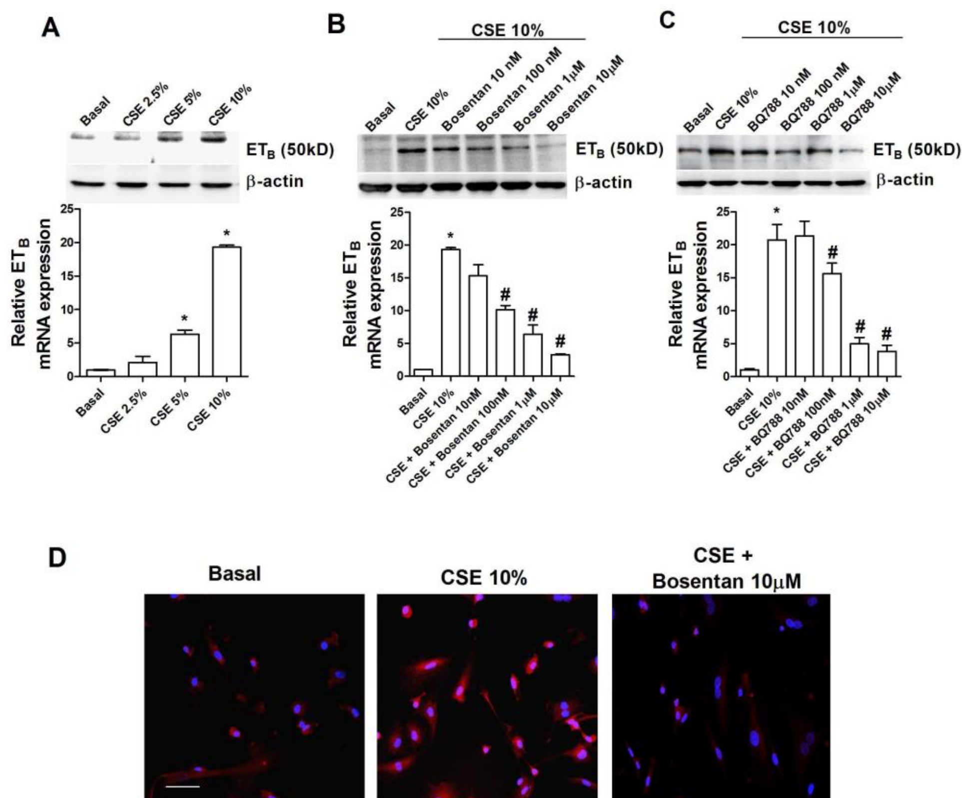

CSE-induced ETB expression in HPAECs is attenuated by bosentan
HPAECs were incubated with different CSE concentrations for 24 h. A) Then, mRNA and protein for ETB were quantified by real time RT-PCR and western blot respectively. B, C) HPAECs were incubated with bosentan (10nM-10μM) or BQ788 (10nM-10μM) 1 h before CSE addition. Bosentan and BQ788 dose-dependently attenuate ETB mRNA and protein expression measured by real time RT-PCR, western blot. D) Immunofluorescence for ETB shows an increment of ETB expression (red color) in cells exposed to CSE which was prevented by bosentan (DAPI-blue colour represents nucleus). Scale bar: 10μm. Results are the mean ±SEM of 4 different experiments from 3 different patients per condition. *P<0.05 vs. basal conditions; #P<0.05 vs. CSE 10%.

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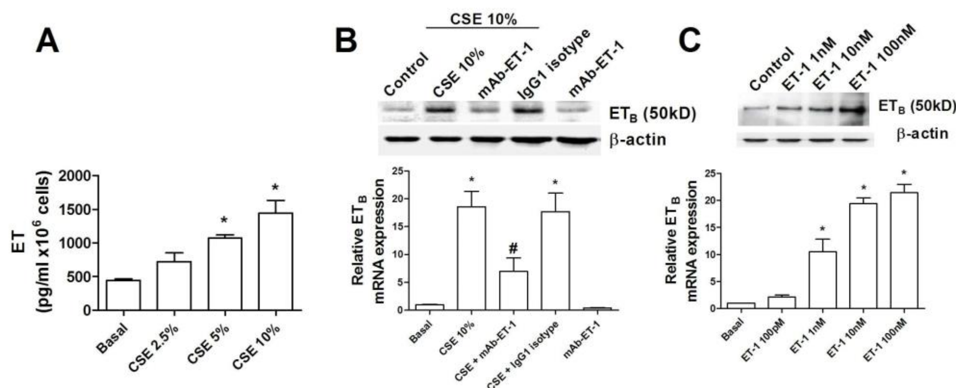

CSE-induced ETB over-expression is partially mediated by supernatant ET levels
A) CSE dose-dependently releases ET to cell culture supernatant after 24 h of CSE-exposure. B) The CSE-induced ETB over-expression was partially inhibited by mAb-ET1 (10µg/ml) and not by the isotype IgG1 control (10µg/ml). C) Furthermore, ET-1 exposure for 24 h, dose-dependently increased ETB mRNA and protein expression. Results are the mean ±SEM of 4 different experiments from 3 different patients per condition. *P<0.05 vs. basal conditions; #P<0.05 vs. CSE 10%.

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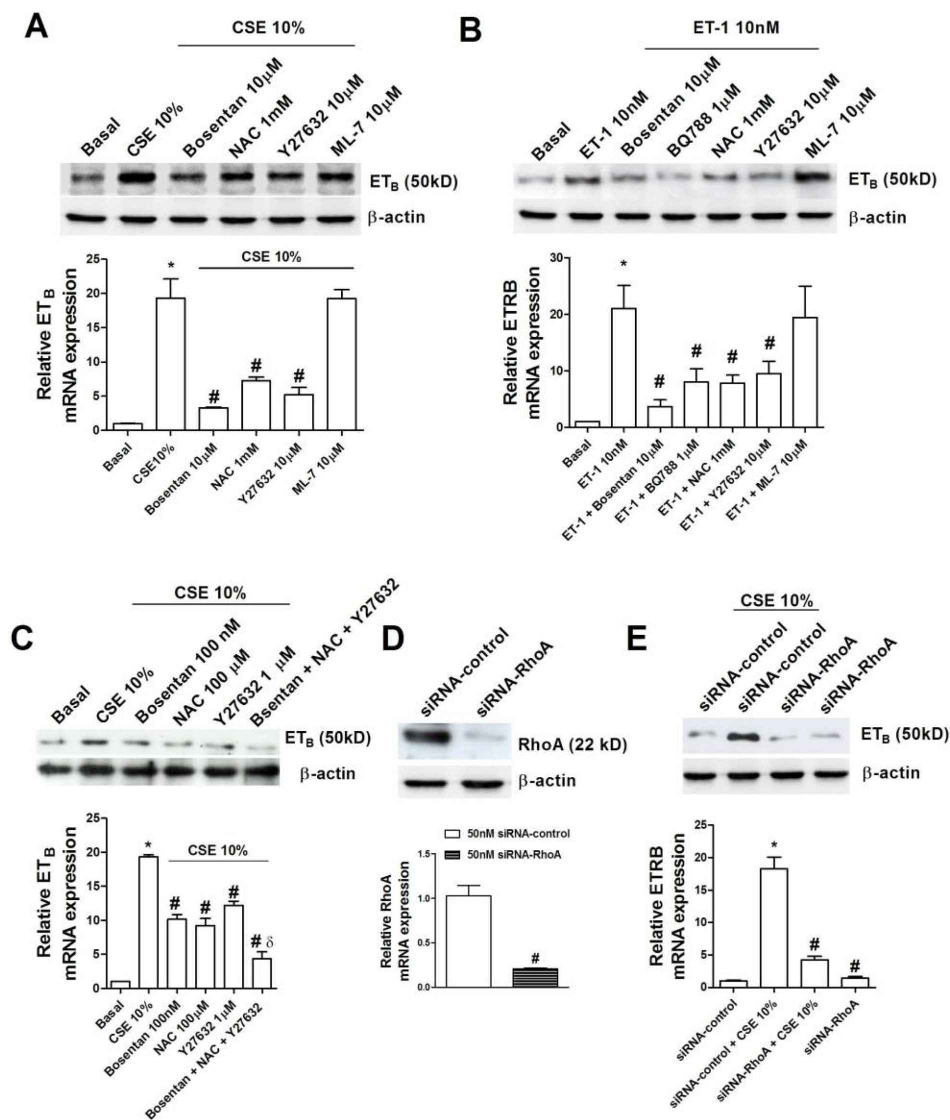

48 CSE-induced ETB over-expression is attenuated by the inhibition of ETB, Rho kinase and ROS
49 A) HPAECs were incubated with bosentan, Y27632, NAC or ML-7 1 h before the addition of CSE 10%. After 24 h of incubation, ETB mRNA and protein were quantified by real time RT-PCR and
50 western blot respectively. B) HPAECs were incubated with bosentan, BQ788, Y27632, NAC or ML-7 1
51 h before the addition of ET-1 (10nM). After 24 h of incubation, ETB mRNA and protein were
52 quantified. C) Additive effects of Bosentan, Y27632 and NAC at ~50% effective concentrations
53 showed additive effects on the inhibition of CSE-induced ETB-up-regulation. D) Specific siRNA
54 targeted to RhoA effectively suppressed RhoA mRNA and protein expression in HPAECs. E) siRNA-
55 RhoA suppressed CSE-induced ETB increase in HPAECs (presented as mRNA and protein expression)
56 respect cells transfected with a negative control siRNA. Results are the mean ±SEM of 3 different
57 experiments from 3 different patients per condition. *P<0.05 vs. control; #P<0.05 vs. CSE 10%.

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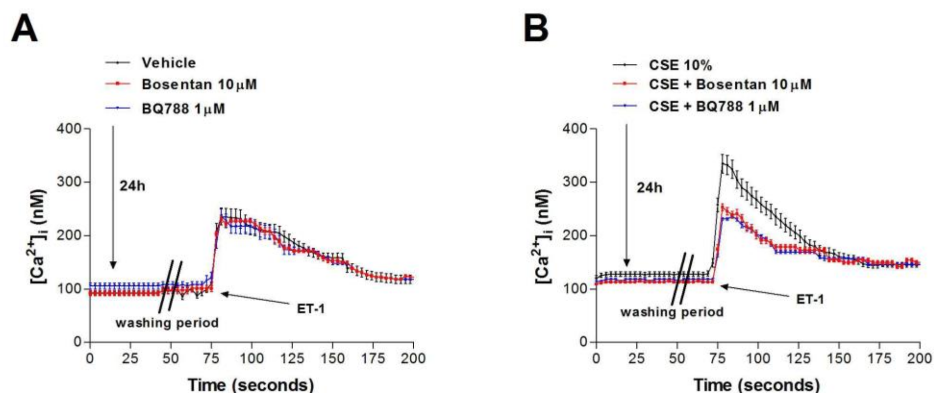

HPAECs exposed to CSE for 24 h increase the acute ET-induced [Ca2+]i
A) HPAECs were exposed to vehicle, bosentan or BQ778 for 24 h. Then cells were washed three times with PBS and incubated with fura 2AM. The increase of [Ca2+]i following acute ET-1 (10nM) was the same for each condition. B) HPAECs were exposed to CSE, in presence or absence of bosentan or BQ778 for 24 h. Then cells were washed three times with PBS and incubated with fura 2AM. The increase of [Ca2+]i following acute ET-1 (10nM) was higher in those cells exposed to CSE 10%. Results are the mean ±SEM of [Ca2+]i nM in 15 cells per experiment in a total of 4 experiments per condition.*P<0.05 vs. control; #P<0.05 vs. CSE 10%.

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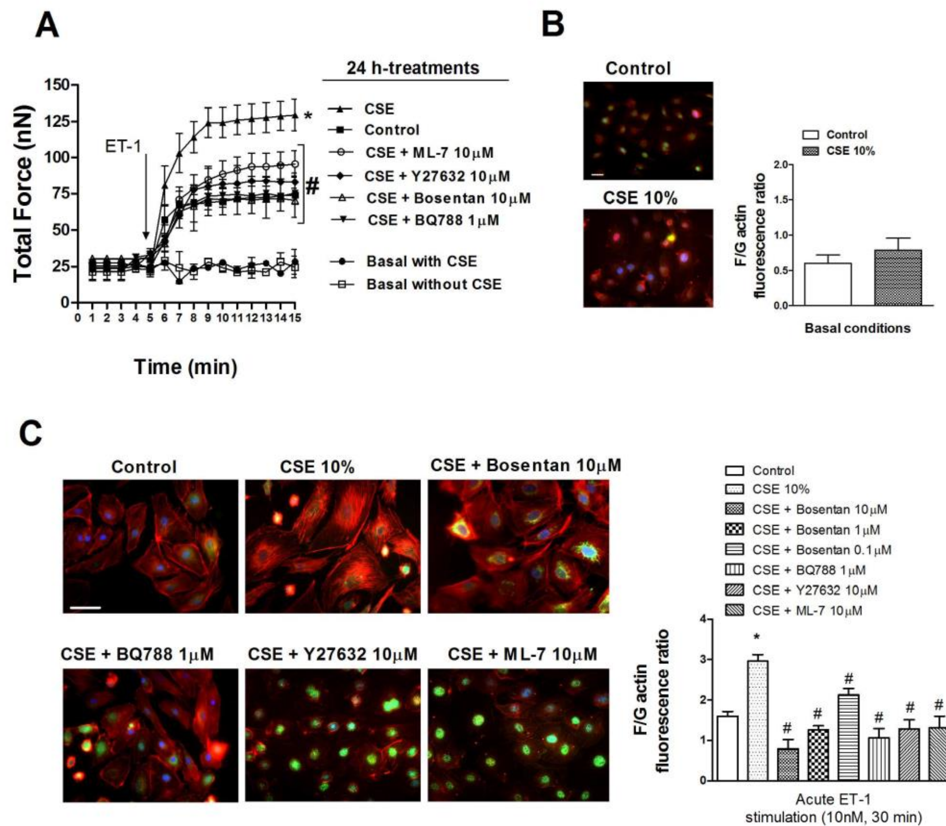

ET-1 increases cell contraction and F/G actin imbalance in HPAECs pretreated with CSE
A) Disks with cultured HPAECs were incubated for 24 h in absence (control) or presence of CSE 10% alone or in combination (1 h before CSE) with bosentan, BQ788, ML-7 or Y27632. Then, gel disk with cultured HPAECs was placed in the microscope and cells imaged with bright-field illumination. Graphic shows the time course of contractile response of HPAECs challenged with ET-1 (10nM). Values are expressed as total force exerted by the cell on the substrate. B) HPAECs were treated with or without CSE for 24 h. Then, cells were fixed with 3.7% formaldehyde and phalloidin-tetramethylrhodamine isothiocyanate and Alexa Fluor 488 DNase I conjugate were added to mark F and G actin respectively. DAPI was added to mark cell nucleus. Images are representative of the F-actin (red colour) and G-actin (green color) staining, and graphic shows the basal F/G actin fluorescence ratio after these experimental conditions. C) In other experiments, bosentan, BQ788, Y27632 or ML-7 were added to cell culture 1 h before CSE. After 24 h of incubation cells were washed three times with PBS and stimulated with ET-1 10nM for 30 min. Then, cells were treated to mark F and G actin. DAPI was added to mark cell nucleus (blue color). HPAECs images show representative experiments under these experimental conditions. Scale bar: 10µm. Graphic represents the mean ±SEM of the fluorescence intensities of F-actin (red) versus G-actin (green) in a total of 3 different experiments from 3 different patients per condition. *P<0.05 vs. control; #P<0.05 vs. CSE.

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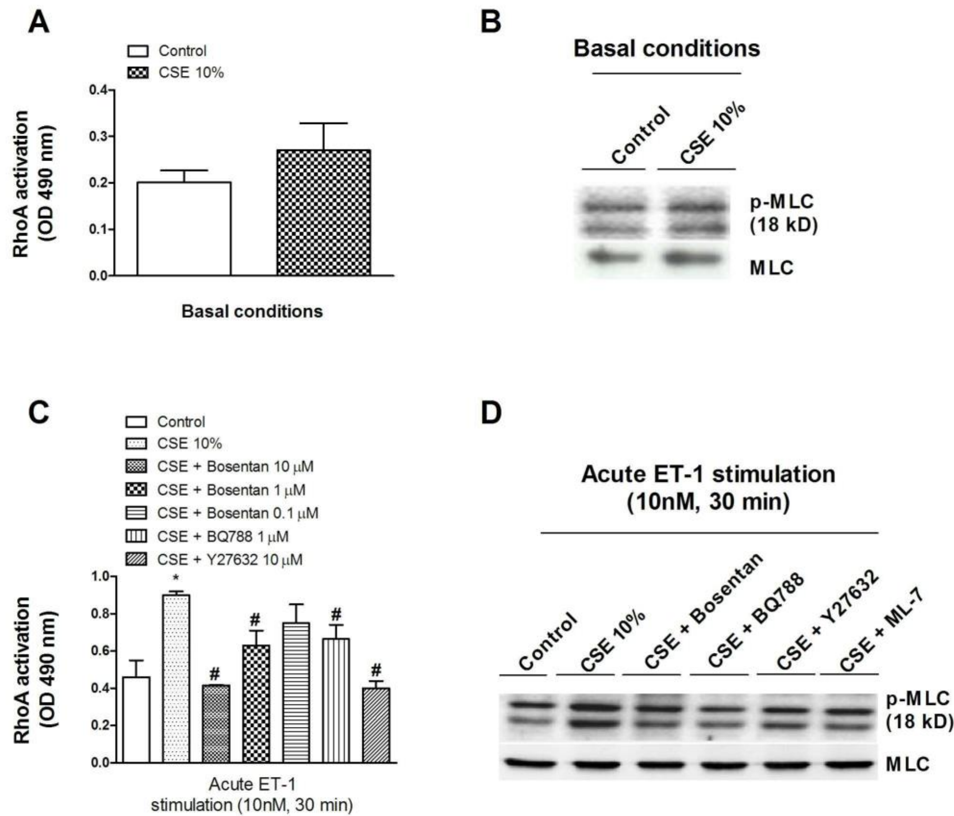

Figure 6

HPAECs exposed to CSE sensitizes cell to ET-1-induced RhoA activation and MLC-phosphorylation. A) RhoA activation was measured in HPAECs treated or not with CSE 10% for 24 h by commercial colorimetric kit. B) After 24 h of CSE exposure MLC-phosphorylation was measured by western blot. C, D) HPAECs were incubated with CSE for 24 h. After CSE treatment cells were washed three times with PBS and stimulated with ET-1 10nM for 30 min, and RhoA activity (C) and MLC phosphorylation (D) were measured. Cells exposed to CSE showed an increase of RhoA activity and p-MLC compared to cells unexposed. Furthermore, the incubation with bosentan, BQ788, Y27632 and ML-7 (only in p-MLC experiments) 1 h before of CSE addition, attenuate the ET-1-induced RhoA activation and MLC phosphorylation. Results are the mean ±SEM of the absorbance corresponding to active RhoA and representative western blot for p-MLC in a total of 3 different experiments from 3 different patients per condition. *P<0.05 vs. control; #P<0.05 vs. CSE 10%.

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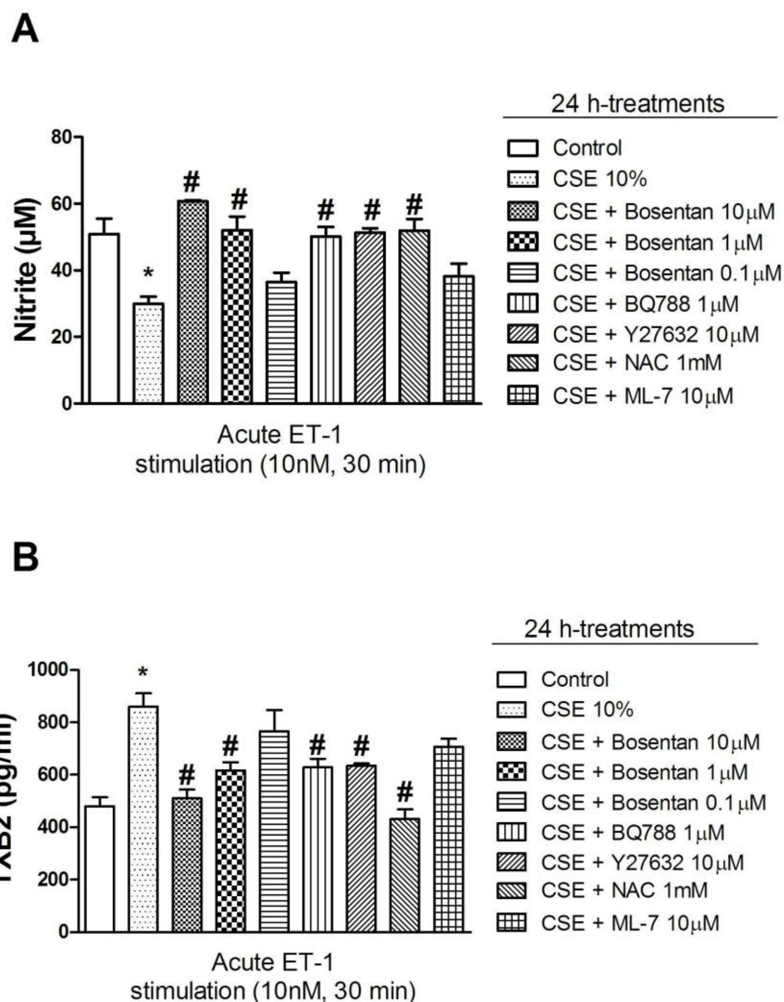

HPAECs exposed to CSE increase the imbalance between NO and TxA2 release in response to acute ET-1.
HPAECs were cultured in 6-well flasks until~95% confluent. Then, cells were incubated with or without CSE 10% for 24 h. In parallel experiments, bosentan, BQ788, Y27632, NAC or ML-7 were added to cell culture 1 h before CSE. After 24 h of incubation, cells were washed three times with PBS and stimulated with ET-1 for 30 min. CSE-treated cells significantly decrease NO release (measured as nitrites) (A) and increase TxA2 release (measured as TxB2) (B) in response to acute ET-1. Furthermore, cell incubation with bosentan, BQ788, Y27632 and NAC attenuated the effect of CSE on ET-1-induced NO decrease and TxA2 increase. Results are the mean ±SEM of a total of 3 different experiments from 3 different patients per condition. *P<0.05 vs. control; #P<0.05 vs. CSE 10%.

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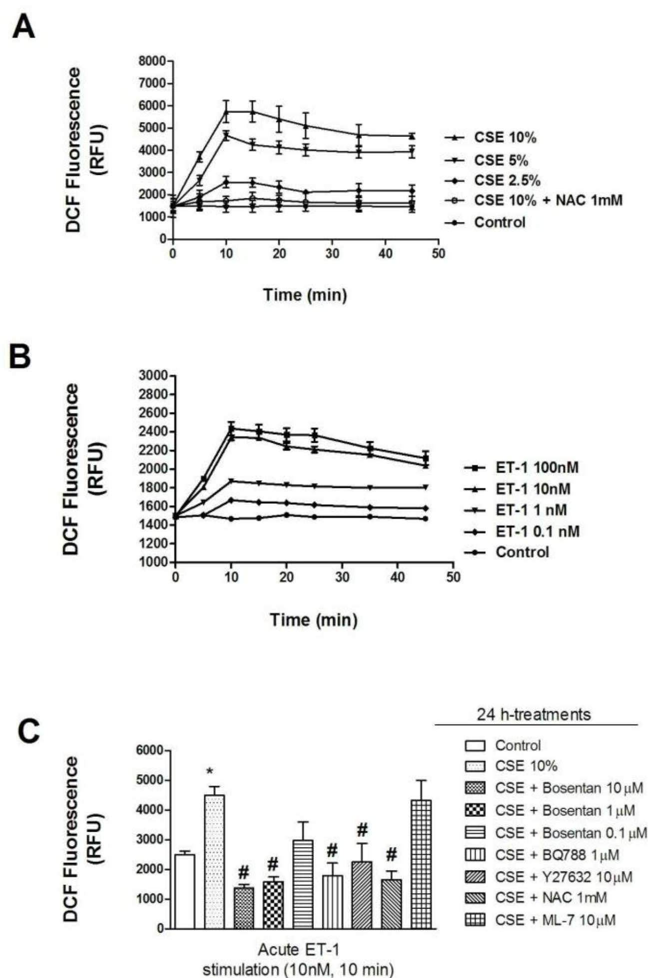

ET-1 increases ROS production in HPAECs treated with CSE
A, B) Confluent HPAEC were cultured in black walled clear bottom 96 well plates and loaded with DCFDA for 30 min. Then, cells were exposed to (A) CSE (2.5%-10%) or (B) ET-1 (0.1-100nM) and DCF fluorescence was monitored each 5 min during 45 min. D) In other experiments, cells were incubated with or without CSE 10% in presence or absence of bosentan, BQ788, Y27632, NAC or ML-7 for 24 h. Then, cells were washed three times with PBS and loaded with DCFDA for 30 min. Thus, cells were stimulated with ET-1 (10nM) and DCF fluorescence was measured after 10 min of stimulation. Results are the mean ±SEM of a total of 6 different experiments from 3 different patients per condition. *P<0.05 vs. control; #P<0.05 vs. CSE 10%.

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Table 2.

HPAECs exposed to CSE for 24 h increase the acute ET-1-induced $[Ca^{2+}]_i$.

HPAECs were incubated for 24 h with or without CSE10%, bosentan, BQ788, and their different combinations. After 24 h, culture medium was removed and cells were washed three times with PBS. Then, cells were loaded with fura-2AM and $[Ca^{2+}]_i$ was monitored following ET-1 (10nM) stimulation. Results are the mean ±SEM of $[Ca^{2+}]_i$ nM or area under curve (AUC) of $[Ca^{2+}]_i$ nM per second in 12 cells per experiment in a total of 4 experiments per condition.*P<0.05 *vs*. control; #P<0.05 *vs*. CSE 10%.

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	Acute ET-1 10 nM stimulation	
24 h pretreatments	**$\Delta[Ca^{2+}]_i$ (nM) (mean ± SEM)**	**AUC ($[Ca^{2+}]_i$ (nM) x seconds) (mean ± SEM)**
Control	142.8 ± 6.3	20730 ± 996.6
CSE 10%	214.5 ± 12.6 *	25056 ± 993.6 *
Bosentan 10µM + CSE 10%	132.8 ± 10.7 #	20221 ± 803.2 #
Bosentan 1µM + CSE 10%	153.8 ± 8.2 #	21311 ± 924.5#
Bosentan 0.1µM + CSE 10%	201.3 ± 11.5	24311 ± 1032.1
BQ788 1µM + CSE	143.5 ± 7.2 #	20855 ± 1090 #
Bosentan 10µM	135.3 ± 6.2	20337 ± 1175
BQ788 1µM	131.7 ± 15.7	20968 ± 488.1

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The authors would like to express their gratitude for the work carried out by the referees in reviewing this manuscript. The revised manuscript has taken into account all the comments and criticisms raised by the reviewers thus improving, to our view, the quality of the revised version.

To ease the review process, in our reply, the reviewer's comments are pasted just preceding the corresponding replies, as indicated. Also, the main changes introduced in the revised manuscript are highlighted in red.

For Peer Review

Reviewer: 1

Comments to the Author

This manuscript reports that CSE upregulates ETb R expression on endothelial cells, and this is associated with increased ET-1 –stimulated signaling and functional effects (increased calcium, RhoA activity, MLC phosphorylation, ROS production). CSE induces ET-1 expression, and ET-1-stimulated NO is suppressed whereas TXA2 is increased. These CSE effects were attenuated by nonselective or selective ETbR antagonists. There is a modest novelty here in that the effects of CSE are shown on endothelial cells; similar effects have been shown in other cells types. The more novel finding is the reversal of effects with an ETbR antagonist, suggesting that some feed-forward effect occurs mediated by ET-1 induction, stimulating ETbR to upregulate its expression and signaling. Whether ET-1 is sufficient to upregulate ETbR expression in endothelial cells is not explored and would contribute to the novelty of this work. In addition, further mechanistic insight could be provided by testing the ability of inhibitors of Rho Kinase, ROS, or PKC to affect such ET-1-induced ETbR upregulation.

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Reply:

We agree with reviewer. At this respect we have performed additional experiments with ET-1 as suggested by the referee. First, we observed that CSE induces ET secretion in HPAECs in a concentration-dependent manner (CSE 2.5% to CSE 10%) after 24h of stimulation as previously outlined (Am J Respir Cell Mol Biol. 2004; Vol 31; 501-509). In this line, we tested whether the ET released by CSE is involved in the CSE-induced ET_B expression. To this respect, we stimulated HPAECs with CSE 10% in presence or absence of human monoclonal antibody against endothelin 1 (mAb-ET-1). The CSE-induced ET_B over-expression was partially inhibited by mAb-ET1 and not by the isotype IgG1 control. Furthermore, direct addition of ET-1 dose-dependently increased ET_B expression (mRNA and protein) confirming the role of ET-1 on ET_B induction. To further investigate this mechanism, we observed that the dual ET receptor antagonist bosentan or the specific ET_B antagonist BQ788 as well as the Rho Kinase inhibitor and ROS inhibitor, significantly attenuated the CSE and ET1-induced ETB up-regulation. In contrast, the MLCK inhibitor did not show any effect. On the other hand, cells treated with siRNA-RhoA effectively prevented the CSE-induced ETB up-regulation. These findings provide new evidence of the mechanism by which CSE increases ETB expression in HPAECs. A statement to this respect has been added to the revised manuscript as suggested by the referee. Methods, page 13, third paragraph. Results, page 16, first and second paragraphs. Figure 2 and figure 3 in revised manuscript.

For Peer Review

Reviewer: 2

1) Several pathways were studied, but none was examined in detail. For example, CSE induction of ETB was blocked by a Rho kinase antagonist and an anti-oxidant, but these

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pathways were not explored further. In this regard, does CSE induce oxidant responses? If so, how are they triggered? This will require further experiments.

Reply:

Reviewer is right. At this respect, we have performed extra experiments, where CSE induces intracellular ROS generation in a time and concentration-dependent manner, reaching a pick value after 10 minutes at 10% concentration following a sustained intracellular increase of ROS. Furthermore, NAC 1mM effectively suppressed the CSE-induced ROS generation. Similar results have been showed previously (FASEB J. 2005; 19(9):1096-107). A statement to this respect has been added to the revised manuscript as suggested by the referee. Methods, page 14, first paragraph. Results, page 20, first paragraph and figure 8 in revised manuscript.

For Peer Review

Reviewer:

2) The reliance on chemical inhibitors is disappointing in view of their non-specific effects and the availability of other strategies including antibodies and siRNA.

Reply:

Reviewer is right. To improve the mechanistic study of this work we employed different strategies. First, we stimulated HPAECs with CSE 10% in presence or absence of human monoclonal antibody against endothelin 1 (mAb-ET1). The CSE-induced ET_B over-expression was partially inhibited by mAb-ET1 and not by the isotype IgG1 control. Furthermore, direct addition of ET-1 dose-dependently increased ET_B expression (mRNA and protein) suggesting a key the role of ET-1 on CSE-induced ETB over-expression. To further investigate this mechanism, we observed that cells treated

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with siRNA-RhoA effectively prevented the CSE-induced ETB up-regulation. These findings provide new evidence of the mechanism by which CSE increases ETB expression in HPAECs. A statement to this respect has been added to the revised manuscript as suggested by the referee. Methods, page 6, second paragraph. Page 8, second paragraph. Page 9, first and second paragraphs. Results, page 16, first paragraph. Page 17, second paragraph and figures 2 and 3 in revised manuscript.

Rviewer

3) The ETB antagonist, BQ788, was used at a dose of 1 uM in some experiments and at 3 uM in others; why is this? Interestingly, no dose-response curves were provided for this agent. Also, I couldn't tell if BQ788 was as effective as bosentan.

Reply:

Reviewer is right. We apologise for this transcription error. BQ788 was used at 1μM concentration in all experiments. Furthermore, we performed additional experiments using different concentrations of BQ788 (10nM to 10μM). In this regard, HPAECs pre-treated with BQ788 dose-dependently abrogates the CSE-induced ETB over-expression. Therefore, BQ788 was as effective as bosentan in preventing ETB over-expression. A statement to this respect has been added to the revised manuscript as suggested by the referee. Results, page 15, third paragraph and figure 1 in revised manuscript.

Reviewer:

Other: The reference list should be carefully revised since some references lack year of publication. Also, it is stated in the text that endothelin was used at 10 nM but Table 2 states 100 nM.

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Reply:

Reviewer is right. We sincerely apologise for these transcription error in the table 2. ET-1, as acute stimulus, was added at 10nM concentration throughout the experiments. Respect the incomplete references, we have added the year of publication. A statement to this respect has been added to the revised manuscript as suggested by the referee. Table 1. Introduction, page 4 third paragraph, page 5, second paragraph, page 10, second paragraph, page 12, second paragraph, page 21, second paragraph, and reference list.

For Peer Review

Reviewer: 3

1. Fig 1: ETB receptor antagonists significantly inhibit the effects of CSE on ETB receptor expression. This suggests a possible feed forward mechanism mediated via ET receptors. The authors may consider measuring ET levels in the supernatant after CSE treatment of the cells.

Reply:

We agree with reviewer. This comment was also suggested by the reviewer 1. We observed that CSE induces ET secretion in HPAECs in a concentration-dependent manner (CSE 2.5% to CSE 10%) after 24h of stimulation which is in line with previous reports (Am J Respir Cell Mol Biol. 2004; Vol 31; 501-509). A statement to this respect has been added to the revised manuscript as suggested by the referee. Methods, page 13, third paragraph. Results, page 16, first paragraphs. Figure 2 in revised manuscript.

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Reviewer:

2. Fig 2: F/G ratio is not a good measure of the cell contractility as immunofluorescence is not a quantitative measurement. Also, what are the baseline F/G ratios (without acute ET stimulation) in control and CSE-treated cells?

Reply:

We agree with reviewer. In this regard, we have conducted cell contraction experiments with the traction microscopy technique as we previously outlined (Chem Biol Interact. 2010 183(1):25-33). We observed that after 24h of CSE-treatment, HPAECs were more sensitive to endothelin-induced contraction compared to those cells without CSE treatment. Furthermore, bosentan, BQ788, Y27632, and ML-7 effectively prevented the effect of CSE on acute ET-1-induced contraction. Respect the baseline F/G ratios (without acute ET stimulation) in control and CSE-treated cells (for 24 h), we did not find any difference between both groups. We have added a representative pictures and graphic in new Figure 5. A statement to this respect has been added to the revised manuscript as suggested by the referee. Results, page 18, third paragraph and figure 5 in revised manuscript.

Reviewer:

3. Fig 3: RhoA activity and MLC phosphorylation studies are very informative. However, it will be interesting to see if CSE treatment alters the basal RhoA activity and MLC phosphorylation. Provide the baseline data on both RhoA activity and MLC phosphorylation.

Reply:

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We agree with reviewer. We have performed additional experiments to provide the basal RhoA activity and MLC phosphorylation as suggested by the referee. We could appreciate a slight increment of both RhoA and MLC phosphorylation after 24 h of CSE exposure that did not reach statistical significance. A statement to this respect has been added to the revised manuscript as suggested by the referee. Results, page 19, first paragraphs and Figure 6 in revised manuscript.

Reviewer:

4. Fig 4: Biochemical measurements of ET levels will be helpful.

Reply:

Experiments from figure 4 were designed to determine the release of NO and TXB2 in response to acute ET-1 to evaluate the effect of the CSE-induced ETB up-regulation in the vasoconstrictor/vasodilator balance. Therefore, the functional consequence of the ETB up-regulation in a context of oxidative stress was the increase in TXB2 and decrease of NO in response to acute ET stimulation. As suggested by the referee we found an increment of ET supernatant levels in response to CSE (10%, 24 h) that are showed in new figure 2. We hope that reviewer understand that the only we wanted to show in figure 4 were the functional effects of ETB up-regulation.

Reviewer:

5. Fig 5: Bosentan, NAC and Y27632 inhibited the expression of ETB receptors in response to CSE. The authors should test if these compounds have additive effects in inducing ETB receptor expression by CSE.

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Reply:

We agree with reviewer. Additional experiments have been done to show potential additive effects between different inhibitors. To this respect, we explored different concentration below maximum effect of each inhibitor. Thus, Bosentan 100nM, Y27632 1μM and NAC 100 μM partially attenuates the CSE-induced ETB-up-regulation. Furthermore, when inhibitors where added together, ETB expression was suppressed almost to control levels showing additive effects. A statement to this respect has been added to the revised manuscript as suggested by the referee. Results, page 16, last paragraph. Page 17, first paragraph and Figure 3 in revised manuscript.

Reviewer:

6. ETB receptor expression data in human samples are very weak. Immunofluorescence is not a quantitative measurement. Increased expression of ETB receptor could be due the fact that there are more PAECs. I am not sure if the data on human subjects is necessary.

Reply:

In response to comments of the reviewer 2 and reviewer 3 we have decided to suppress data from human subjects. We understand that the immunofluorescence is more qualitative than quantitative technique, and not provide decisive information to this mechanistic manuscript.

Reviewer:

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7. Table 2: the authors need to provide representative tracings of the calcium responses in control and treated PAECs. Were there any changes in the baseline intracellular calcium levels in these cells upon treatment with CSE?

Reply:

Representative tracings of the intracellular calcium levels and the responses in control and treated HPAECs are showed in new figure 4. The baseline intracellular calcium concentration in control group was 93±3.4nM significantly lower than the baseline intracellular calcium concentration for cells treated with CSE 10% (120.2±4.6 nM; $P<0.05$ vs. control). A statement to this respect has been added to the revised manuscript as suggested by the referee. Results, page 17, third paragraph and figure 4 in revised manuscript.

For Peer Review

Minor:

1. There are several typos and grammatical mistakes throughout the manuscript.
2. Figure numbers in the text are not correct (at least in the printed version I read).
3. Discussion is too long.

Reply:

Manuscript has been revised for grammatical mistakes, figure numbers have been corrected and discussion has been shortened.