BJP BRITISH JOURNAL OF PHARMACOLOGY
BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00979.x
www.brjpharmacol.org

RESEARCH PAPER

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

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

Received
16 March 2010
Revised
20 June 2010
Accepted
16 July 2010

BACKGROUND AND PURPOSE

Pulmonary arteries from smokers and chronic obstructive pulmonary disease 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 in endothelial dysfunction.

EXPERIMENTAL APPROACH

ET_B receptor expression was measured by real time RT-PCR, Western blot and immunofluorescence. Cell contraction, intracellular Ca^{2+}, F/G-actin, RhoA activity, myosin light chain phosphorylation, ET, NO, thromboxane (Tx)A_2 and reactive oxygen species (ROS) were measured by traction microscopy, fluorescence microscopy, phalloidin fluorescence, colorimetric assay, Western blot, ELISA and DCFDA fluorescence respectively.

KEY RESULTS

Cigarette smoke extract dose-dependently increased ET_B receptor expression in HPAECs after 24 h incubation. CSE-induced ET_B expression was attenuated by bosentan, the ET_B receptor antagonist BQ788, the Rho kinase antagonist Y27632 and the antioxidant N-acetylcysteine. A monoclonal antibody to ET-1 prevented CSE-induced ET_B receptor overexpression. Twenty-four hour exposure to ET-1 dose-dependently increased ET_B receptor expression, mimicking the effect of CSE. CSE-induced ET_B receptor overexpression caused greater cell contraction; increased intracellular Ca^{2+}; increased F/G-actin and RhoA activity; increased myosin light chain phosphorylation; augmented TxA_2 and ROS production; and decreased NO after acute ET-1 (10 nM). These effects were attenuated by bosentan, BQ788, Y27632 and N-acetylcysteine.

CONCLUSIONS AND IMPLICATION

Cigarette smoke extract induced ET_B receptor overexpression by a feed forward mechanism mediated partly by ET release, promoting HPAEC dysfunction and attenuated by ET_B receptor blockade, Rho kinase and ROS inhibition. These results provide support for the use of bosentan in CS-related endothelial dysfunction.

Abbreviations

BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular free calcium concentration; CSE, cigarette smoke extract; DCF, 2′, 7′-dichlorofluorescein; DCFDA, 2′, 7′-dichlorofluorescein diacetate; FCS, fetal calf serum; HPAECs, human pulmonary artery endothelial cells; mAb, monoclonal antibody; NAC, N-acetylcysteine; PBS, phosphate-buffered saline; PH, pulmonary hypertension; ROS, reactive oxygen species

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 and Cool, 2003). Pulmonary arteries of COPD patients show abnormal endothelium-dependent vascular reactivity (Dinh-Xuan *et al.*, 1991; Peinado *et al.*, 1998). Moreover, the endothelial function of pulmonary arteries in healthy smokers lies between that in non-smokers and COPD patients, indicating that endothelial dysfunction is present at early stages 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 progressive changes in the vascular structure and function that underlie the development of PH in COPD.

Many studies have shown the deleterious effect of CS on endothelial function (Santos *et al.*, 2002; Wright *et al.*, 2004; Milara *et al.*, 2010) coupled with the expression and release of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α. (Wright *et al.*, 2004; Orosz *et al.*, 2007; Milara *et al.*, 2010). Furthermore, CS promotes endothelial cell contraction through the activation of RhoA and myosin light chain (MLC) kinase leading to increased endothelial cell permeability, which is an important component in the pathogenesis of PH (Bernhard *et al.*, 2005; Morrell *et al.*, 2009; Richens *et al.*, 2009). CS also impairs nitric oxide (NO) release from endothelial cells, caused by CS-derived reactive oxygen species (ROS) inactivating endothelial NO synthase (eNOS) via the interaction of Rho kinase with eNOS (Sugimoto *et al.*, 2007). Moreover, products derived from CS decrease vasodilators and anti-mitogenic agents such as NO and prostacyclin (PGI_2) and increase the release of vasoconstrictors and pro-mitogenic agents, such as endothelin-1 (ET-1) and thromboxane A_2 (TxA_2) thereby promoting endothelial dysfunction and vascular remodelling (Barua *et al.*, 2003; Wright *et al.*, 2006; Nana-Sinkam *et al.*, 2007).

Endothelin-1 is a secreted peptide that signals through two transmembrane G protein-coupled receptors, ET_A and ET_B (receptor nomenclature follows Alexander *et al.*, 2009). These receptors are both simultaneously expressed in all cell types studied, with one notable exception; only the ET_B receptor is expressed on endothelial cells (Migneault *et al.*, 2005).

Under normal conditions endothelial ET_B receptors elicit vasodilatation and anti-mitogenic effects through the release of NO and/or PGI_2 in pulmonary endothelial cells and, to a lesser extent, mediate release of ET-1 and TxA_2 (Galie *et al.*, 2004). In addition, pulmonary ET_B receptors also mediate the pulmonary clearance of circulating ET-1 and the reuptake of ET-1 by endothelial cells (Dupuis *et al.*, 1996). Significantly, the role of ET_B receptors in PH induced by hypoxia is well established, as mice with specific knockout of the endothelial cell ET_B receptors present an exaggerated increase in right ventricular pressure during hypoxia (Kelland *et al.*, 2010). However, CS-induced PH in COPD patients exhibits characteristics significantly different from those found in primary hypoxaemic PH patients. In this context, the number of inflammatory cells infiltrating the wall of pulmonary arteries correlates with the enlargement of the intimal layer and endothelial dysfunction (Peinado *et al.*, 1999). Furthermore, recent studies have shown that the systemic inflammation associated with COPD appears to increase the risk for developing PH in humans as well as in *in vitro* and *in vivo* models of pulmonary artery remodelling induced by tobacco smoke (Eddahibi *et al.*, 2006; Joppa *et al.*, 2006; Stenmark *et al.*, 2006). Interestingly, ET_A and ET_B receptors are up-regulated in bronchial biopsies from COPD patients and ET-1 is up-regulated in COPD patients with PH (Moller *et al.*, 1999; Carratu *et al.*, 2008), which suggest that changes in the ET signalling system may not be limited to an increased production of ET-1. Furthermore, it has been shown recently that CS mediates the up-regulation of ET_A and ET_B in rat bronchial and vascular smooth muscle cells, thereby increasing their contractility (Granstrom *et al.*, 2006; Xu *et al.*, 2008). However, no data have been reported concerning the effect of CS on ET_B receptor expression and the functional consequences in endothelial cells.

Therefore, we hypothesized that CS, the main agent involved in COPD and the subsequent

endothelial dysfunction, may affect ET_B receptor expression in human pulmonary artery endothelial cells (HPAECs), and that this modulation could influence HPAEC function. Moreover, we decided to evaluate bosentan [a putative therapeutic agent in the treatment of some forms of PH in COPD (Barbera and Blanco, 2009)], a dual ET_A-ET_B receptor antagonist, on the endothelial cell function following CS-induced expression of ET_B receptors *in vitro*.

Methods

Isolation and culture of HPAECs

With the approval of the local ethics committee and informed consent of the patients, peripheral human lung tissue was obtained from 15 non-smoker patients (3 women, 12 men (65 ± 4 years) who were undergoing surgery for lung carcinoma. None of the patients exhibited clinical evidence of PH or COPD, and none was chronically treated with theophylline, β-adrenoceptor agonists, corticosteroids or anti-cholinergic drugs. Only macroscopically normal segments of artery were used for our experiments.

We used HPAECs from non-smokers in order to maximize the *in vitro* effects of CS. 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% fetal calf serum (FCS), and the homogenate was centrifuged at 250× *g* for 10 min at 4°C.

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 and Murray, 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 CD31-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 CD31-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 CD31-coated Dynabeads were discarded.

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 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. n°: Hs00240747_m1; RhoA: cat. n^a: Hs00357608_m1) and GAPDH (pre-designed by Applied Biosystems, cat. n°: 4352339E) as a housekeeping gene 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 small interfering RNA

Small interfering RNA (siRNA), including the scrambled siRNA control, were purchased from Ambion (Huntingdon, Cambridge, UK). RhoA-targeted siRNA (ID: s758) sense 5′-CACAGUGUUU GAGAACUAUtt-3′ and antisense 5′-AUAGUUCUCA AACACUGUGgg-3′ were designed from Ambion. The HPAECs were transfected with siRNA (50 nM) in serum- and antibiotic-free medium, over 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 2 $\mu L \cdot mL^{-1}$. 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. n° MCA5312Z). Protein expression was referred to β-actin (1:1000, Sigma, USA) expression as internal control. All experiments were performed in triplicate.

Western blot

Western blot analysis was used to detect changes in ET_B receptors (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 EDTA (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 receptor (1:1000) antibody (polyclonal antibody, cat. n°: E9905, Sigma, UK), rabbit anti-human diphospho-MLC (Thr18/Ser19) and rabbit anti-human MLC (1:1000) antibodies (rabbit polyclonal antibodies, cat. n°: 3674 and 3672, Cell signalling), monoclonal mouse anti-human RhoA (1:1000) antibody (AbD serotec, UK, cat. n° MCA5312Z) or β-actin (1:1000) as control, followed by a peroxidase conjugated secondary (1:10 000) antibody. The 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 previously reported (Ortiz *et al.*, 2009; Milara *et al.*, 2010). 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 puffs·min^{-1}; 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. A dilution to 10% CSE 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. In order 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).

Measurement of intracellular free Ca^{2+}

Intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured by epifluorescence microscopy (Nikon TE200, Tokyo, Japan) in HPAECs using the Ca^{2+} indicator dye fura-2 as previously outlined (Dalli *et al.*, 2008). In brief, fluorescence of fura-2 acetoxymethyl ester (5 μM)-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, 1300 × 1030 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 convert the 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 340 and 380 nm, 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 subtracted for each experiment. Minimum and maximum fluorescence intensities were obtained with the addition of Ca^{2+}-free solution with 10 mM EDTA solution and the Ca^{2+} ionophore ionomycin 10 μM in presence of 5 mM $CaCl_2$ solution, respectively, as previously outlined (Dalli *et al.*, 2008; Cortijo *et al.*, 2010). The experiments performed on intracellular Ca^{2+} were designed to study the functional effect of the overexpression of ET_B receptors induced by CSE.

Actin staining

Cells were washed three times with 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 PBS for 3–5 min and washed

again with PBS. Phalloidin-tetramethylrhodamine isothiocyanate (0.2 μg·mL^{-1}) and Alexa Fluor 488 DNase I conjugate (9 μg·mL^{-1}) were used to localize F-actin and G-actin, respectively, as described by 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 ×10 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 ×40 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 polyacrylamide 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, a gel disk with cultured HPAECs was placed in the microscope and cells examined 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 gel substrate (Cortijo *et al*., 2010). Measurements were taken in 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 with the G-LISA protocol, using the 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

Endothelin 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.

Fluorescence measurement of ROS

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^- and H_2O_2, and can therefore be used to monitor 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 versus time (min).

Immunofluorescence

Human pulmonary artery endothelial cells 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 receptor (1:100, cat. n°: E9905, Sigma) overnight at 4°C, followed by secondary antibody anti-rabbit rhodamine (1:100, Molecular Probes) and DAPI (2 μg/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 overexpression of ET_B receptors induced by CSE. For this, HPAECs were treated with or without CSE for 24 h in presence or absence of bosentan or different pharmacological modulators (added 1 h before CSE). Then, HPAECs were washed three times with PBS and stimulated with exogenous ET-1 (10 nM) for different times to mimic the lung microenvironment associated with PH where ET is highly elevated (Holm and Franco-Cereceda, 1996; Cacoub *et al.*, 1997). At the end of the ET-1 stimulation, the variables described above were measured and compared with corresponding values from cells not exposed to CSE.

Statistical analysis

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, USA). Every experiment showed in this study was performed in HPAECs from at least three different patients in three to four experiments per patient. Significance was accepted when $P < 0.05$.

Materials

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 sulphoxide as 10 mM stock solutions. Several dilutions of the stock solutions were prepared, using cell culture medium. The final concentration of dimethyl sulphoxide 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. n°: ab20940) was used at 10 $\mu g \cdot mL^{-1}$ concentration to suppress the effect of ET-1 released into cell supernatants, as previously outlined (Didier *et al.*, 2003). Non-specific mouse IgG1 was used at the same concentration as control.

Results

CSE-induced up-regulation of ET_B receptors in HPAECs is partly suppressed by bosentan

Cigarette smoke extract dose-dependently increased ET_B receptor mRNA and protein expression in HPAECs after 24 h of incubation (Figure 1A). Interestingly, the overexpression of ET_B receptors induced by CSE was inhibited by bosentan in a concentration-dependent manner (10 nM–10 μM; Figure 1B), reaching statistical significance at 100 nM (Figure 1B, $P < 0.05$ vs. CSE 10%). In addition, the selective ET_B receptor antagonist BQ788 also reduced, in a concentration-dependent manner, the ET_B receptor overexpression (10 nM–10 μM; Figure 1C), suggesting a feed forward mechanism mediated via ET receptors. These results were also confirmed by immune-fluorescence staining with ET_B receptor antibody (Figure 1D). Incubation with bosentan or BQ788 alone did not show any effect on basal ET_B receptor expression (data not shown). To elucidate further the mechanisms involved in the CSE-induced ET_B overexpression, the amount of ET in cell culture supernatants were measured. 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 to ET-1 (10 $\mu g \cdot mL^{-1}$), significantly reduced the overexpression of ET_B receptors induced by CSE suggesting that the ET-1 released by CSE was involved in this process (Figure 2B, $P < 0.05$ vs. CSE 10%). Interestingly, stimulation of HPAECs with exogenous ET-1 dose-dependently (100 pM–100 nM) also increased ET_B receptor expression. These results demonstrate that ET-1 is one of the downstream effectors of CSE causing ET_B receptor overexpression.

Overexpression of ET_B receptors induced by CSE is partly mediated by the activation of ET_B and the downstream pathway Rho kinase and ROS production

As CS is known to promote RhoA and MLCK activation and ROS production, we studied the effect of these downstream pathways on the overexpression of ET_B receptors induced by CSE. The Rho kinase inhibitor, Y27632 (10 μM) and the antioxidant NAC (1 mM) effectively reversed the stimulation of mRNA and protein for ET_B receptors induced by CSE (Figure 3A). In contrast, the MLCK inhibitor ML-7 did not show any significant effect (Figure 3A). When ET-1 (10 nM) was used to induce ET_B receptor expression, bosentan (10 μM), BQ788 (1 μM), Y27632 (10 μM), the antioxidant NAC (1 mM) but not ML-7 partly suppressed the ET_B receptor overexpression (Figure 3B) suggesting a role for ET-1 in CSE-induced ET_B receptor up-regulation.

In other experiments we explored the possible additive effects of bosentan, Y27632 and NAC. We selected concentrations of bosentan (100 nM), Y27632 (1 μM) and NAC (100 μM), which caused ~50% inhibition of the maximal effect of CSE (Figure 3C). The combination of these three inhibitors showed an additive effect in reducing the ET_B receptor up-regulation, following CSE (Figure 3C, $P < 0.05$ vs. bosentan, Y27632 and NAC groups).

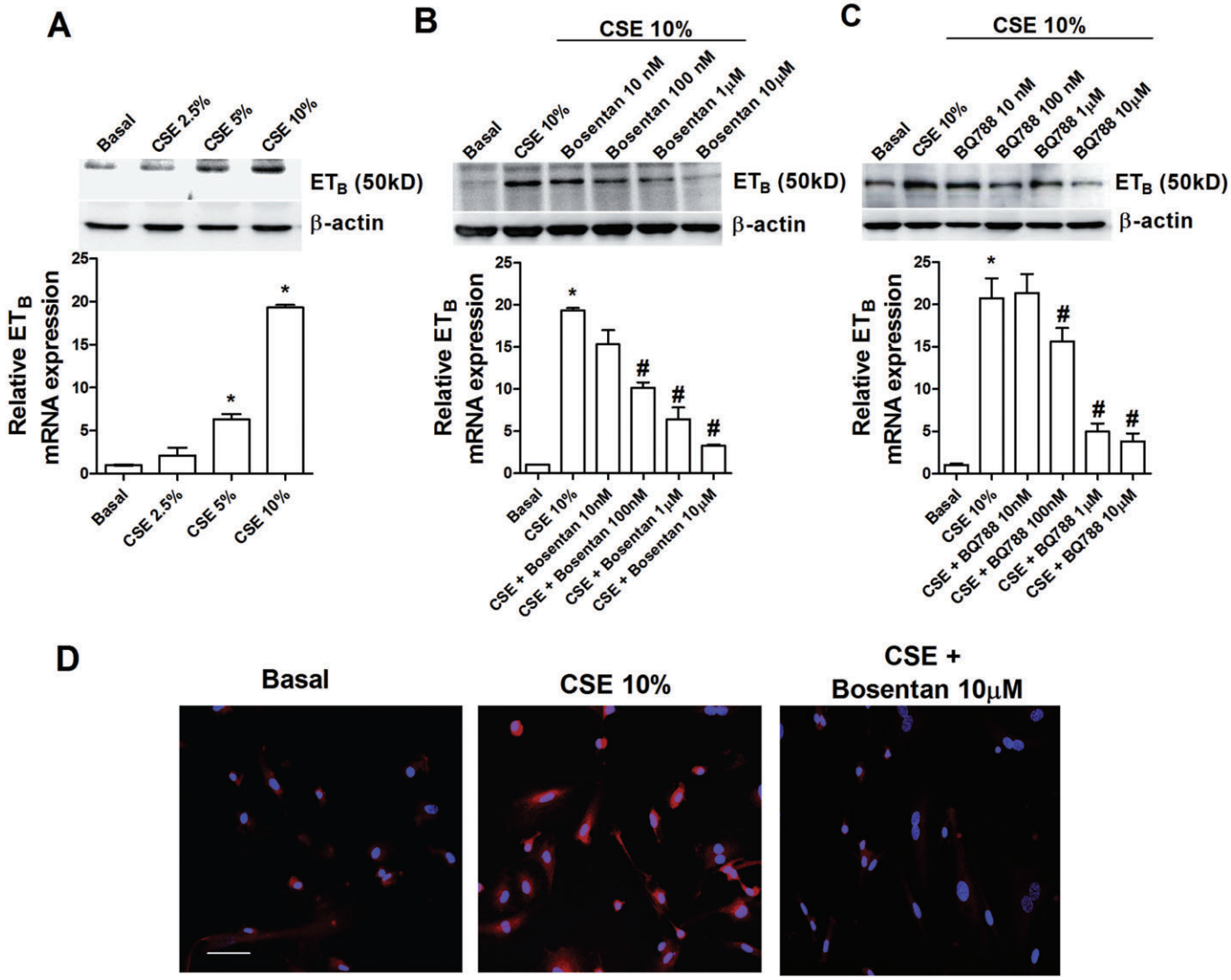

Figure 1

Cigarette smoke extract (CSE)-induced ET_B expression in human pulmonary artery endothelial cells (HPAECs) is attenuated by bosentan. HPAECs were incubated with different CSE concentrations for 24 h. (A) Then, mRNA and protein for ET_B receptors were quantified by real time RT-PCR and Western blot respectively. (B, C) HPAECs were incubated with bosentan (10 nM–10 μM) or BQ788 (10 nM–10 μM) 1 h before CSE addition. Bosentan and BQ788 dose-dependently attenuated ET_B receptor mRNA and protein expression, as measured by real time RT-PCR and Western blot. (D) Immunofluorescence for ET_B receptors showed an increment of ET_B receptor expression (red colour) in cells exposed to CSE, which was prevented by bosentan (DAPI-blue colour represents nucleus). Scale bar: 10 μm. Results are the mean ± SEM of four different experiments from three different patients per condition. *$P < 0.05$ versus basal conditions; #$P < 0.05$ versus CSE 10%.

In order to investigate further the role of RhoA in our model, we selectivity antagonized 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). This treatment also prevented the CSE from inducing overexpression of ET_B receptors, confirming the results observed with Y27632 (10 μM) (Figure 3E, $P < 0.05$ vs. siRNA control plus CSE 10%).

Exposure of HPAECs to CSE increased sensitivity to raised $[Ca^{2+}]_i$ induced by ET-1

$[Ca^{2+}]_i$ modulates several cellular process such as endothelial cell contraction and permeabilization, proliferation and inflammation (Garcia *et al.*, 1993). As CSE increases ET_B receptor expression, we investigated the effect of acute exposure to exogenous ET-1 on $[Ca^{2+}]_i$ in HPAECs pretreated for 24 h with or without CSE in presence or absence of bosentan

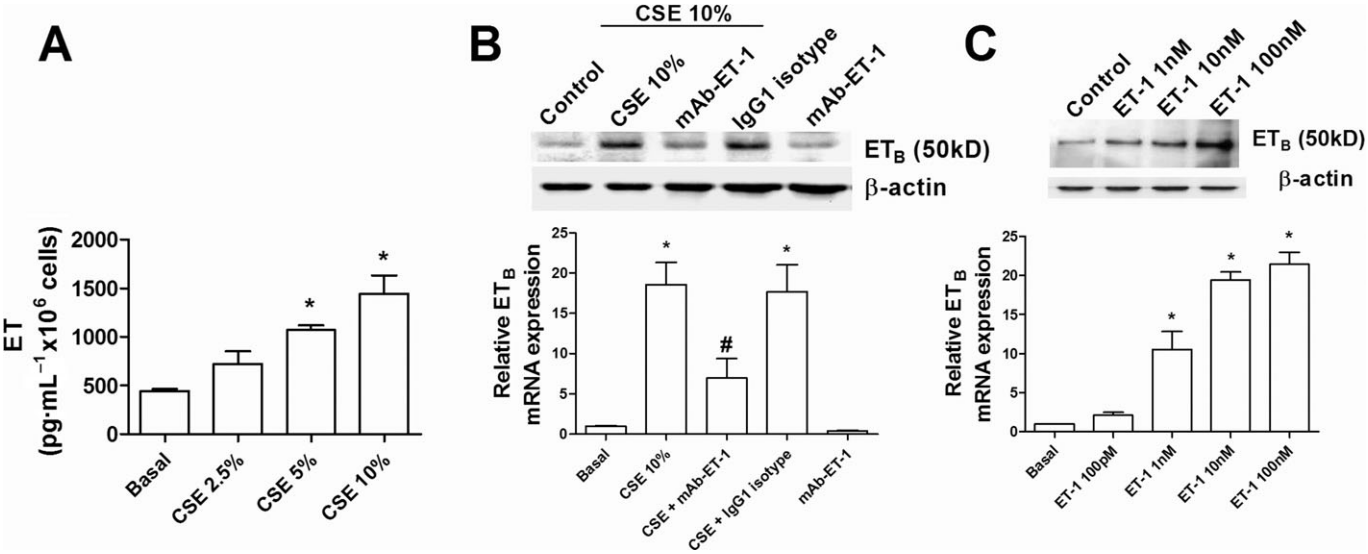

Figure 2

Cigarette smoke extract (CSE)-induced ET_B receptor overexpression is partly mediated by ET in cell supernatants. (A) CSE dose-dependently releases ET to cell culture supernatant after 24 h of CSE exposure. (B) CSE-induced ET_B receptor overexpression was partly inhibited by mAb to ET-1 (10 μg·mL^{-1}) and not by the isotype IgG1 control (10 μg·mL^{-1}). (C) Furthermore, incubation of human pulmonary artery endothelial cells (HPAECs) with ET-1 for 24 h dose-dependently increased ET_B receptor mRNA and protein expression. Results are the mean ± SEM of four different experiments from three different patients per condition. *P < 0.05 versus basal conditions; #P < 0.05 versus CSE 10%.

(10 μM) or BQ788 (1 μM). Preliminary experiments showed that ET-1 increased $[Ca^{2+}]_i$ in HPAECs in a dose-dependent manner ($-\log EC_{50}$ = 7.62 ± 0.06, data not shown). Therefore, a concentration of 10 nM ET-1 was used in subsequent experiments. HPAECs stimulated with CSE (10%) for 24 h showed a $[Ca^{2+}]_i$ baseline of 120 ± 5 nM, which is significantly higher than that observed for unstimulated cells ($[Ca^{2+}]_i$ baseline of 93 ± 3 nM; P < 0.05). Furthermore, stimulation with ET-1 (10 nM, over 3–4 min) increased $[Ca^{2+}]_i$ (shown as $\Delta[Ca^{2+}]_i$) in CSE-treated cells more than in untreated cells (P < 0.05) (Figure 4A,B and Table 1). In other experiments, bosentan (0.1–10 μM) or BQ788 (1 μM) was added 1 h before incubation with 10% CSE, for a further 24 h. Under these conditions, bosentan (1–10 μM) and BQ788 (1 μM) significantly reduced the effect of subsequent stimulation with exogenous ET-1 (10 nM) on $[Ca^{2+}]_i$ (Figure 4A,B and Table 1, P < 0.05).

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

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

Cytoskeletal rearrangement is a direct marker of cell contraction and polymerization of soluble G-actin to F-actin fibres is a part of such rearrangement (Gavara *et al.*, 2006). The F/G-actin fluorescence ratio value of HPAECs exposed to CSE 10% for 24 h was not different from that in cells without CSE (Figure 5B). In contrast, the CSE-treated cells showed an increased F/G-actin ratio in response to acute stimulation with ET-1 (10 nM; 30 min) (Figure 5C, P < 0.05). The addition of bosentan (0.1–10 μM), BQ788 (1 μM), Y27632 (10 μM) or ML-7 (10 μM) 1 h before CSE (10%) significantly reduced this increase of F/G-actin ratio induced by ET-1 (Figure 5C, P < 0.05 vs. CSE).

Because Y27632 and ML-7 attenuated the effects of CSE on ET-1-induced cell contraction, we explored the activation of RhoA and the phosphorylation of MLC in those cells exposed to CSE. In HPAECs, the basal RhoA activity and MLC phosphorylation was not affected by CSE (10%; 24 h; Figure 6A,B). However, exogenous ET-1 (10 nM, 30 min) increased RhoA activity more in cells exposed to CSE, than in cells without CSE (Figure 6C, P < 0.05). Furthermore, when bosentan (1 and 10 μM), BQ788 (1 μM) and Y27632 (10 μM) were added before CSE (for 24 h), the ET-1-induced

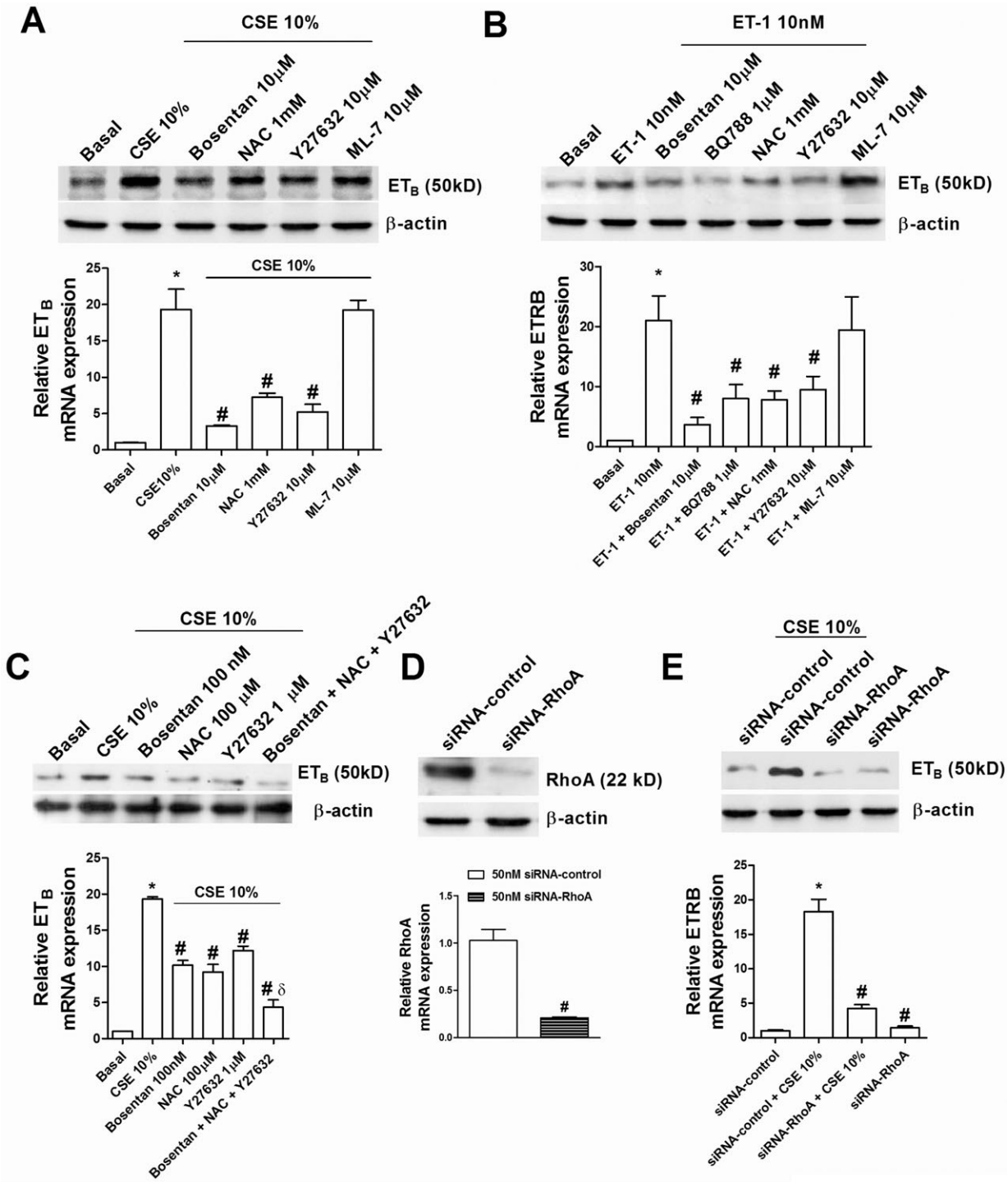

Figure 3

Cigarette smoke extract (CSE)-induced ET_B receptor overexpression is attenuated by the blockade of ET_B receptors, Rho kinase or reactive oxygen species (ROS). (A) Human pulmonary artery endothelial cells (HPAECs) were incubated with bosentan, Y27632, NAC or ML-7 for 1 h before the addition of CSE 10%. After 24 h of incubation, ET_B receptor 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 (10 nM). After 24 h of incubation, ET_B receptor mRNA and protein were quantified. (C) Additive effects of bosentan, Y27632 and NAC at ~50% effective concentrations showed additive effects on the inhibition of CSE-induced ET_B receptor up-regulation. (D) Specific siRNA targeted to RhoA effectively suppressed RhoA mRNA and protein expression in HPAECs. (E) siRNA-RhoA suppressed the overexpression of ET_B receptors induced by CSE in HPAECs (presented as mRNA and protein expression), compared with cells transfected with a negative control siRNA. Results are the mean ± SEM of three different experiments from three different patients per condition. *$P < 0.05$ versus control; #$P < 0.05$ versus CSE 10%.

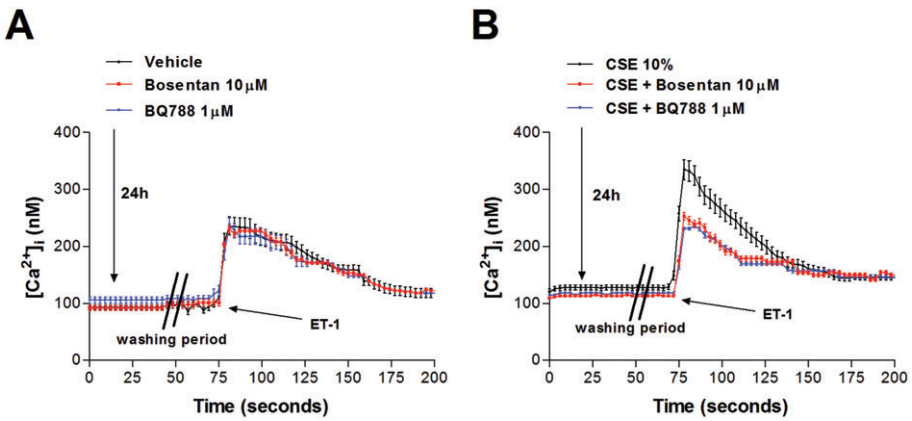

Figure 4

Exposure of human pulmonary artery endothelial cells (HPAECs) to cigarette smoke extract (CSE) for 24 h increased the rise in $[Ca^{2+}]_i$ induced by ET-1. (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 stimulation with ET-1 (10 nM), was the same for each condition. (B) HPAECs were exposed to 10% 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 (10 nM) was higher in the cells exposed to 10% CSE. Results are the mean ± SEM of $[Ca^{2+}]_i$ nM in 12 cells per experiment in a total of four experiments per condition.*$P < 0.05$ versus control; #$P < 0.05$ versus CSE 10%.

Table 1

Exposure of HPAECs to CSE for 24 h increased the rise in $[Ca^{2+}]_i$ induced by acute stimulation by exogenous ET-1

24 h pretreatment	Acute ET-1 10 nM stimulation $\Delta[Ca^{2+}]_i$ (nM)	AUC [$[Ca^{2+}]_i$ (nM) × seconds]
Control	143 ± 6	20730 ± 997
CSE 10%	215 ± 13*	25056 ± 994*
Bosentan 10 μM + CSE 10%	133 ± 11#	20221 ± 803#
Bosentan 1 μM + CSE 10%	154 ± 8#	21311 ± 925#
Bosentan 0.1 μM + CSE 10%	201 ± 12	24311±1032
BQ788 1 μM + CSE 10%	144 ± 7#	20855±1090#
Bosentan 10 μM	135 ± 6	20337 ± 1175
BQ788 1 μM	132 ± 16	20968 ± 488

HPAECs were incubated for 24 h with or without CSE 10%, 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 stimulation with ET-1 (10 nM). Results are the mean ± SEM of the rise in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$; nM) or area under curve (AUC; nM × seconds) of $\Delta[Ca^{2+}]_i$ in 12 cells per experiment in a total of four experiments per condition.
*$P < 0.05$ versus control; #$P < 0.05$ versus CSE 10%.
HPAECs, human pulmonary artery endothelial cells; CSE, cigarette smoke extract.

RhoA activity was decreased (Figure 6C, $P < 0.05$ vs. CSE). Similarly, CSE (10%) increased the effect of ET-1 on MLC phosphorylation and this was attenuated by bosentan (10 μM), BQ788 (1 μM), Y27632 (10 μM) or ML-7 (10 μM) (Figure 6D).

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 have been reported concerning the influence of CS on ET-1-induced NO and TxA_2 release. As CSE increased ET_B receptor expression, the effects of ET-1 on NO and TxA_2 release could be different. Therefore, HPAECs were exposed to CSE for 24 h and were then stimulated with ET-1 (10 nM) for 1 h. After stimulation, the stable derivatives of NO and TxA_2, namely nitrites and TxB_2, were measured in the cell culture supernatant. We observed that CSE treatment reduced the ET-1-induced nitrite release by ~50% compared with untreated cells (Figure 7A, $P < 0.05$). Furthermore, addition of bosentan (1 and 10 μM),

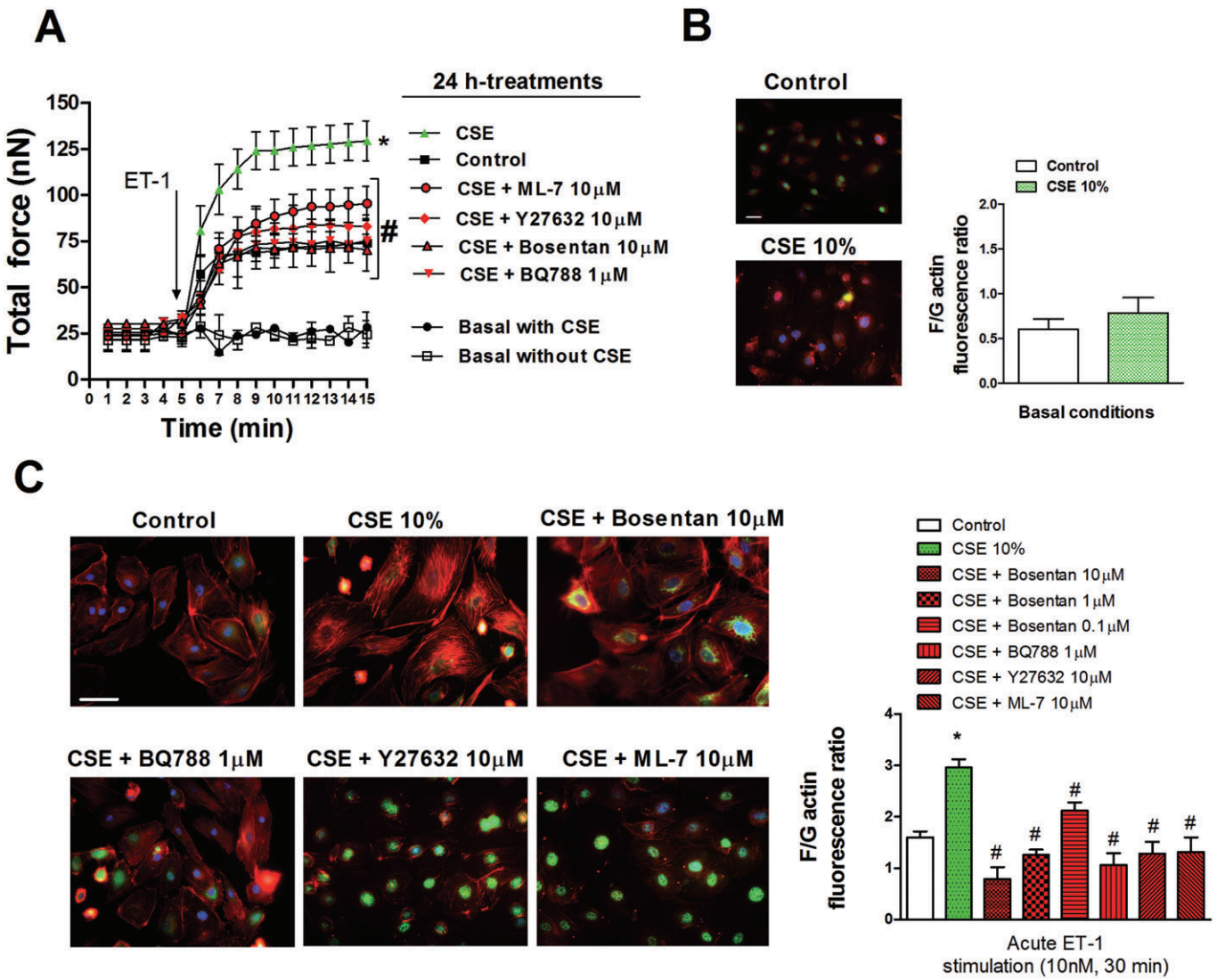

Figure 5

ET-1 increases cell contraction and F/G-actin imbalance in human pulmonary artery endothelial cells (HPAECs) pretreated with cigarette smoke extract (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, a gel disk with cultured HPAECs was placed in the microscope and cells imaged with bright-field illumination. The graph shows the time course of contractile response of HPAECs challenged with ET-1 (10 nM). 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 colour) staining, and the graph 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 10 nM for 30 min. Then, cells were treated to mark F- and G-actin. DAPI was added to mark cell nucleus (blue colour). HPAECs images show representative experiments under these experimental conditions. Scale bar: 10 μm. The graph shows the mean ± SEM of the fluorescence intensities of F-actin (red) versus G-actin (green) in a total of three different experiments from three different patients per condition. *$P < 0.05$ versus control; #$P < 0.05$ versus CSE.

BQ788 (1 μM), Y27632 (10 μM) or the antioxidant NAC (1 mM) significantly increased nitrite release following ET-1 (Figure 5A, $P < 0.05$ vs. CSE). In contrast, ML-7 (10 μM) failed to modify NO release. Conversely, in HPAECs treated with CSE, TxB_2 release induced by ET-1, was increased by approximately twofold (Figure 5B, $P < 0.05$ vs. unexposed cells to CSE). Bosentan (1 and 10 μM), BQ788 (1 μM), Y27632 (10 μM) and NAC (1 mM) significantly reduced the TxB_2 release by ET-1 (Figure 5A, $P < 0.05$ vs. CSE), while ML-7 did not show any effects.

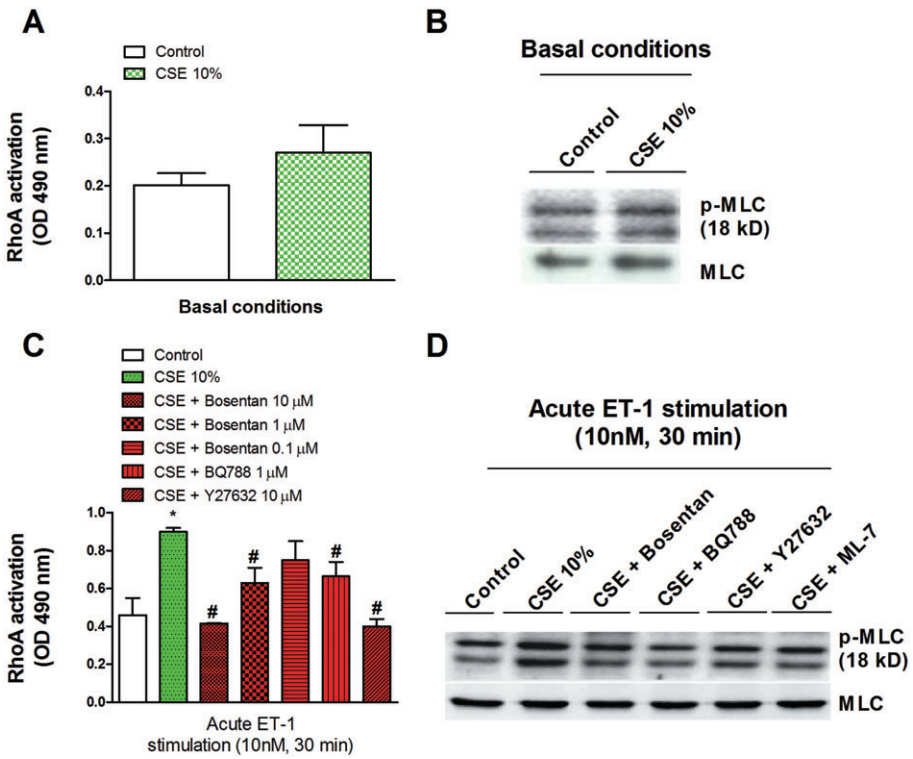

Figure 6

Exposure of human pulmonary artery endothelial cells (HPAECs) to cigarette smoke extract (CSE) sensitizes cells to ET-1-induced RhoA activation and myosin light chain (MLC) phosphorylation. (A) RhoA activation was measured in HPAECs, with or without CSE treatment (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 10 nM 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 with cells not exposed. Furthermore, incubation with bosentan, BQ788, Y27632 or ML-7 (only in p-MLC experiments) 1 h before CSE addition, attenuated 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 three different experiments from three different patients per condition. *$P < 0.05$ versus control; #$P < 0.05$ versus CSE 10%.

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

Reactive oxygen species are purported to be the main initiator of intracellular reactivity in endothelial cells exposed to CS. Thus, we examined endothelial ROS production in response to CSE and found that CSE increased intracellular ROS in a time and concentration-dependent manner, reaching a peak value after 10 min of stimulation (Figure 8A). This maximum was followed by a subsequent decline that plateaued above baseline levels after ~30 min (Figure 8A). The antioxidant NAC (1 mM) completely prevented ROS generation (Figure 8A). Second, we studied the role of CSE on ROS production induced by exogenous ET-1. As ET-1 (10 nM) induced submaximal 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 exogenous ET-1, which was significantly higher than in HPAECs not exposed to CSE (Figure 6C, $P < 0.05$ vs. unexposed cells). When bosentan (1 and 10 μM), BQ788 (1 μM), Y27632 (10 μM) and NAC (1 mM) were added to HPAECs before CSE, the effect of ET-1 on ROS production was suppressed (Figure 8C, $P < 0.05$ vs. CSE).

Discussion

This study was designed to evaluate the effect of CS on ET_B receptor expression in HPAECs and to examine the functional consequences in the context of ET-1 exposure *in vitro*. We found for the first time that HPAECs stimulated with CSE increase ET_B receptor expression via a feed forward mechanism involving ET release from CSE-exposed cells. Moreover, bosentan as well as the selective ET_B receptor inhibitor BQ788 prevented the overexpression of ET_B receptors induced by CSE. Furthermore, such overexpression sensitized HPAECs to ET-1

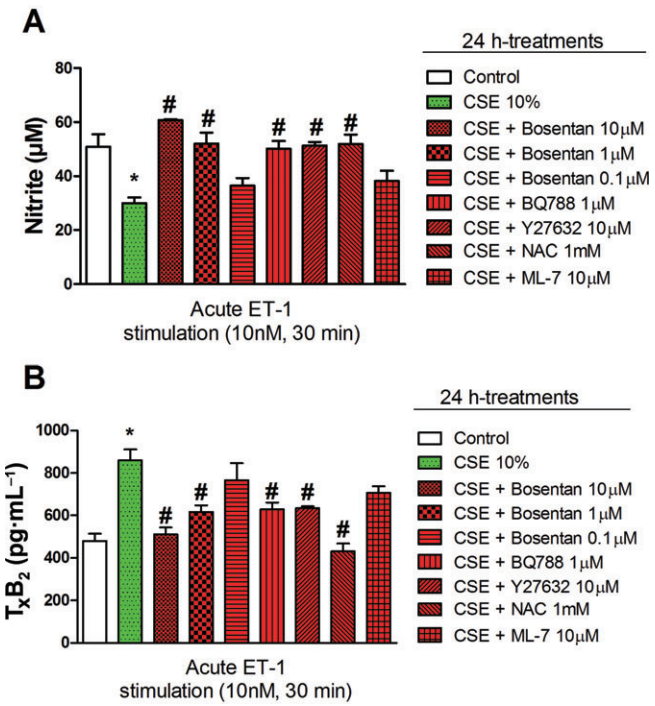

Figure 7

Exposure of human pulmonary artery endothelial cells (HPAECs) to cigarette smoke extract (CSE) increased the imbalance between NO and TxA_2 release in response to acute ET-1. HPAECs were cultured in six 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 cultures, 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 released less NO (measured as nitrites) (A) and more TxA_2 (measured as TxB_2) (B), in response to acute ET-1. Furthermore, cell incubation with bosentan, BQ788, Y27632 or 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 three different experiments from three different patients per condition. *$P < 0.05$ versus control; #$P < 0.05$ versus CSE 10%.

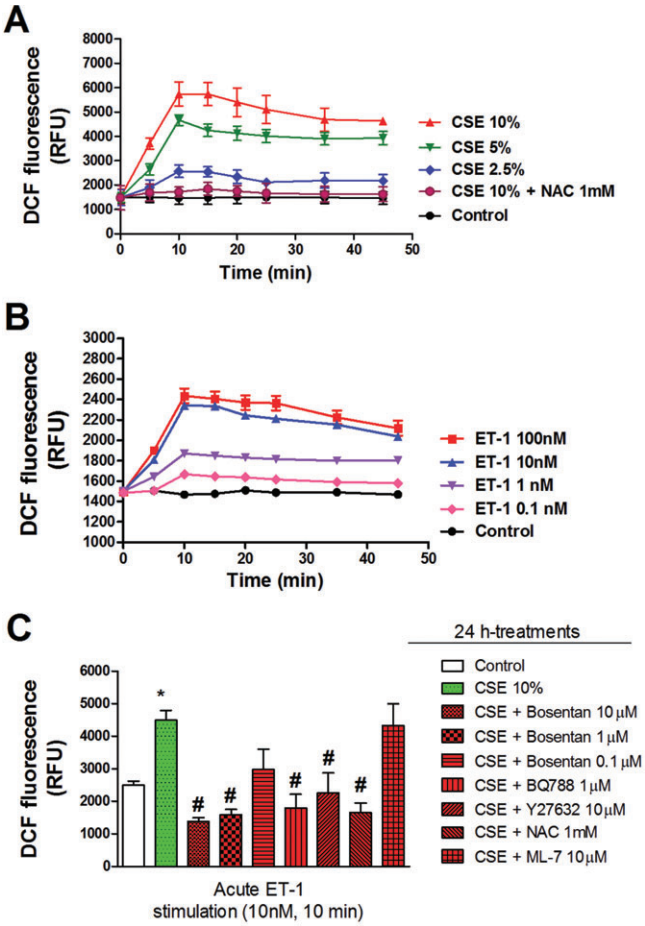

Figure 8

ET-1 increases reactive oxygen species (ROS) production in human pulmonary artery endothelial cells (HPAECs) treated with cigarette smoke extract (CSE). (A, B) Confluent HPAECs 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 (0.1–100 nM) and DCF fluorescence was monitored every 5 min during 45 min. (C) 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. Cells were stimulated with ET-1 (10 nM) and DCF fluorescence was measured after 10 min of stimulation. Results are the mean ± SEM of a total of six different experiments from three different patients per condition. *$P < 0.05$ versus control; #$P < 0.05$ versus CSE 10%.

stimulation, thereby causing endothelial dysfunction, as shown by increased cell contraction; reduced NO synthesis and increased production of TxA_2 and of ROS. These results suggest that CS exerts pro-inflammatory effects by the up-regulation of ET_B receptors, potentiating endothelial dysfunction induced by ET-1.

Recent studies focussing on ET_B receptor function in endothelial cells suggest a protective role, as its stimulation released vasodilators and anti-angiogenic factors such as NO and PGI_2. Moreover, it has been shown that endothelial ET_B receptor knockout mice develop severe right ventricular pressure during hypoxia (Kelland *et al.*, 2010), and that endothelial ET_B receptors remove ET-1 from the systemic circulation into lung tissue. In contrast to ET_A receptors, the ET_B receptors are inducible proteins in both smooth muscle cells and endothelial cells (Galie *et al.*, 2004; Yeligar *et al.*, 2009). However, in thromboembolic PH and in PH derived from systemic sclerosis as well as in inflammatory conditions, ET_B receptor expression is up-regulated mainly in pulmonary arterial smooth muscle cells where ET_B receptors mediate contraction and smooth muscle cell proliferation (Bauer *et al.*, 2002; Frommer and Muller-Ladner, 2008) thereby contributing to pulmonary remodelling. Furthermore, CS also up-regulates ET_B receptor expression in

pulmonary artery smooth muscle cells causing pulmonary artery contraction (Xu *et al.*, 2008). However, the role of CS on ET_B receptor expression and its functional consequences in endothelial cell function remain to be elucidated.

In this work we focused on the effect of CS as an inflammatory stimulus, which could be a primary agent in the initial endothelial damage that could induce PH in COPD patients. We found that CSE induced ET_B receptor overexpression in HPAECs and that this was prevented by the ET_B/ET_A receptor antagonist bosentan, the selective ET_B receptor 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 (Sugimoto *et al.*, 2007; Richens *et al.*, 2009; Milara *et al.*, 2010), and that ROS generation may up-regulate ET_B receptor expression (Yeligar *et al.*, 2009). As CSE induces ET release (Wright *et al.*, 2006), and the activation of ET_B receptors induces ROS production (Dong *et al.*, 2005), it seems reasonable to suggest that bosentan and BQ788 may inhibit the overexpression of ET_B receptors induced by CSE. Indeed, we detected an increased ET concentration following CSE exposure. Furthermore, the addition of mAb to ET-1 partially suppressed the CSE-induced ET_B receptor overexpression. This finding was reinforced by evidence that ET_B receptors were up-regulated by ET-1, suggesting that the CSE-induced overexpression of ET_B receptors is mediated, in part, by a feed forward mechanism.

Although the expression of ET_B receptors in endothelial cells is thought to be associated with beneficial effects, its overexpression in an inflammatory context has not been fully examined. Thus, we investigated the functional role of the overexpression of ET_B receptors induced by CSE in HPAECs. Endothelial permeability may be an important component of the pathogenesis of PH, because it increases infiltration of inflammatory cells into the wall of pulmonary arteries, thereby enlarging the intimal layer and promoting pulmonary remodelling. In this context, it has been suggested that the development of structural and functional abnormalities in pulmonary arteries of patients with COPD is related to inflammatory cell invasion of the vascular wall by inflammatory cells (Wright *et al.*, 2005).

The GTPase RhoA and the myosin kinase MLCK play a key role in cell contraction and permeability (Oka *et al.*, 2008). Stimulation of ET_B receptors induces RhoA activation and intracellular Ca^{2+} release, and both mediate MLC phosphorylation, which increases formation of F-actin stress fibres, cell contraction and permeability (Oka *et al.*, 2008; Morrell *et al.*, 2009). Consistent with these findings, *in vivo* studies have shown that selective ET_B receptor activation increased albumin extravasation in guinea pig lungs (Filep *et al.*, 1995). In this work, we observed that CSE-induced overexpression of ET_B receptors in HPAECs sensitizes these cells to acute stimulation by exogenous ET-1. Thus, CSE-exposed cells showed an increased effect of acute ET-1 in responses associated with cell permeability such as the increase of $[Ca^{2+}]_i$, RhoA activation, MLC phosphorylation, F-actin polymerization and cell contraction. As pre-incubation with bosentan, BQ788 and Y27632 reduced these effects and prevented the ET_B overexpression, we would suggest that ET-1-induced endothelial permeability in cells exposed to CSE was mediated, in part, by the induction of ET_B receptor expression.

Next, we focused on the effect of exposure to CSE in terms of NO and TxA_2 release induced by ET-1. The balance of the vasodilator/vasoconstrictor cell release is a key regulator of endothelial cell function. Under physiological conditions, HPAECs mainly release the vasodilator and anti-angiogenic agents, NO and PGI_2. However, during cell injury, this balance may be altered thereby promoting the release of vasoconstrictors (Christman *et al.*, 1992). There is strong evidence that part of the ET-1-induced vasoconstriction is mediated by increased TxA_2 production, which, in turn, is mediated by the ET_B receptors in the vascular endothelium (D'Orleans-Juste *et al.*, 1994; Curzen *et al.*, 1995). The endothelial ET_B receptors 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 treatment of HPAECs with CSE sensitized these 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, suggesting that ET_B receptors, Rho kinase and ROS were involved in this process.

It is well established that CS inhibits 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 vasodilator/vasoconstrictor release. Thus, ET-1-induced ROS production was highest in HPAECs exposed to CSE suggesting that ET_B receptor overexpression increased ROS production. Furthermore, bosentan, BQ788, Y27632 and

NAC completely suppressed the effect of CSE on ET-1-induced ROS. As these compounds also prevented the overexpression of ET_B receptors secondary to CSE, we would postulate that the ET-1-induced ROS in CSE-exposed cells is mediated, at least in part, by ET_B receptor overexpression.

In summary, we would like to propose a model in which CSE induces ET_B receptor overexpression by a feed forward mechanism mediated in part by release of ET from the cells, promoting HPAEC dysfunction, which is partly attenuated by blockade of ET_B receptors, Rho kinase and ROS. These results may provide *in vitro* evidence supporting the use of bosentan in CS-related endothelial dysfunction.

Acknowledgements

This work was supported by grants SAF2008-03113 (JC), SAF2009-08913 (EJM), CIBERES (CB06/06/0027) from Ministry of Science and Innovation and Health Institute 'Carlos III' of Spanish Government, and research grants (Prometeo/2008/045) from Regional Government ('Generalitat Valenciana').

Conflicts of interest

This work was partly supported by Actelion Pharmaceuticals Ltd, Switzerland who provided bosentan.

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