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

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ABSTRACT: The endothelin (ET) system contributes to lung vascular tension and remodelling in smokers and chronic obstructive pulmonary disease (COPD) patients.

This study examined the effect of cigarette smoke (CS) on ET receptor A (ETA) and B (ETB) expression in human pulmonary artery smooth muscle cells (HPASMCs) and human small intrapulmonary arteries, as well as their functional consequences.

CS extract (CSE) increased ETA and ETB expression in HPASMCs and small intrapulmonary arteries, which was attenuated by bosentan, the ETA antagonist BQ123 and the ETB antagonist BQ788, and by blocking ET-1 with a monoclonal antibody against ET-1, suggesting a feed-forward mechanism mediated by ET-1 release. ET receptor (ETR) antagonism attenuated the CSE-induced HPASMC proliferation. Furthermore, CSE exposure increased the acute ET-1-induced small intrapulmonary artery contraction, which was attenuated by bosentan, BQ123 and BQ788. Pulmonary arteries from smokers and COPD patients showed a higher expression of ETA and ETB than those of nonsmoker patients.

These results show a novel mechanism by which ETR blockade attenuates CS-induced ETR overexpression and, subsequently, small intrapulmonary artery tension. These data may be of potential value to explain therapeutic effects of bosentan in some forms of disproportionate pulmonary hypertension in COPD patients.

KEYWORDS: Bosentan, cigarette smoke, endothelin receptor, human pulmonary artery smooth muscle cells, precision-cut lung slice

Pulmonary hypertension (PH) is a relatively common form of cigarette smoke (CS)-induced lung disease. PH develops in ~6% of subjects with chronic obstructive pulmonary disease (COPD) but is present in ~40% of patients with a forced expiratory volume in 1 s of <1 L [1, 2]. The pathogenesis of PH in COPD is unclear. Current studies suggest that PH is caused by the direct effects of CS on the intrapulmonary vessels by the secretion of a number of vasoconstrictive/proliferative peptides, such as endothelin (ET) or vascular endothelial growth factor, which subsequently contribute to vascular remodelling and the development of PH [3]. The circulating levels of ET-1 are elevated after exposure to CS in humans [4] and it has been shown that ET-1 correlates with pulmonary systolic pressure in COPD patients with PH [5], suggesting that ET-1 is involved in CS-induced

vascular remodelling. ET-1 activates two receptors, ETA and ETB, which are located in pulmonary artery smooth muscle cells (PASMCs), whilst exclusively ETB are present in endothelial cells. Both ETA and ETB mediate proliferation and contractility in PASMCs from small pulmonary arteries, while endothelial ETB mediates vasodilation in normal pulmonary arteries. ETB upregulation has been observed in human blood vessels from patients with ischaemic heart disease [6], hypertension [7] and severe PH [8]. More recently, it has been shown that CS extract (CSE) induces ETA and ETB overexpression in resistant cerebral arteries from rats by a mechanism implicating the activation of the intracellular mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase (ERK)1/2 and p38, the transcription factor nuclear factor (NF)-κB, and c-Jun N-terminal kinase (JNK) [9–11].

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The data suggest that the upregulation of ET receptors (ETRs) is an important molecular mechanism that could play an essential part in the development of pathological lung arteries secondary to CS, such as intimal thickening, vessel narrowing and PH. Therefore, therapeutic interventions focused on inhibiting ETR expression could be of potential value to ameliorate pulmonary artery remodelling and tension in smokers with COPD. Currently, no data exist concerning the effect of CS on ETR expression in human PASMCs (HPASMCs) and human small intrapulmonary arteries, and on the effect of ETR antagonists on the regulation of ETR.

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

METHODS

See the online supplement for further details of the methods used.

Patients

A total of six nonsmoker controls, seven smokers and eight COPD patients were included in the study. All lung tissues studied were taken from uninvolved lung tissue during lobectomy resection for malignant lesions. Samples of distal lung, located as far away as possible from the tumour, were chosen for the study. Pulmonary function tests (forced spirometry) and arterial blood gas measurements were performed during the days prior to surgery. None of the patients exhibited clinical evidence of PH. HPASMCs for *in vitro* experiments were isolated from 2–3-mm pulmonary arteries of nonsmoker lung tissue. Precision-cut lung slices with small intrapulmonary arteries (internal diameter 100–300 µm) were prepared from nonsmoker lung tissue. Isolated pulmonary arteries of internal diameter 0.5–1.0 mm were used to measure ETR expression in nonsmokers, smokers and COPD patients. The protocol was approved by the local research and independent ethics committee of the University General Hospital of Valencia (Valencia, Spain). Informed written consent was obtained from each participant. Clinical features of patients are defined in table 1.

Isolation and culture of HPASMCs

Tumour-free material from surgical specimens of nonsmoker patients was used. HPASMCs were isolated from surgical specimens of human pulmonary arteries as previously outlined [12]. Briefly, segments of pulmonary artery (internal diameter 2–3 mm) were digested with 1% collagenase (Gibco, Paisley, UK) in RPMI-1640 culture medium for 30 min at 37°C. HPASMCs were isolated from human pulmonary artery endothelial cells (HPAECs) by means of CD31-coated Dynabeads (Dynal Biotech, Darmstadt, Germany) as previously outlined [12] and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf

TABLE 1 Clinical features

	Nonsmokers	Smokers	COPD
Subjects n	6	7	8
Age yrs	66±5	68±7	67±9
Tobacco consumption pack-yrs	0	30±3	40±8
FEV₁ % pred	92±3	90±6	68±8
FVC % pred	94±4	93±8	87±6
FEV₁/FVC %	89±5	87±7	63±5
TLC %pred	90±6	88±3	97±4
P_aO₂ mmHg	90±8	85±7	84±6
P_aCO₂ mmHg	37±6	39±3	41±3

Data are presented as mean±SEM, unless otherwise stated. COPD: chronic obstructive pulmonary disease; FEV₁: forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity; TLC: total lung capacity; P_aO₂: arterial oxygen tension; P_aCO₂: arterial carbon dioxide tension.

serum, 1% fungizone and 2% streptomycin/penicillin. All HPASMCs studied were used from passage 1 to 4.

Real time RT-PCR

Real-time RT-PCR was performed as previously outlined [13]. In brief, total RNA was isolated from cultured HPASMCs using TriPure® Isolation Reagent (Roche, Indianapolis, IN, USA). cDNA was amplified with specific primers for ETB and ETA (pre-designed by Applied Biosystems, Foster City, CA, USA; ETB: catalogue number Hs00240747_m1; ETA: catalogue number Hs03988672_m1) and glyceraldehyde phosphate dehydrogenase (GAPDH) (pre-designed by Applied Biosystems; catalogue number 4352339E) as a housekeeping gene. Relative quantification of these different transcripts was determined with the 2^{-ΔΔCt} method using GAPDH as the endogenous control and normalised to the control group.

Western blot

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

Preparation of CSE solutions

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

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

Intracellular free calcium measurements

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

Cell contraction

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

Cell proliferation assay

HPASMC proliferation was measured by colorimetric immunoassay based on bromodeoxyuridine (BrdU) incorporation during DNA synthesis using a cell proliferation ELISA BrdU kit (Roche, Mannheim, Germany) as previously outlined [14].

Rho activity and ET assays

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

Dichlorofluorescein diacetate fluorescence measurement of reactive oxygen species

Intracellular reactive oxygen species (ROS) levels (H_2O_2 and superoxide anion) were measured in HPASMCs by means of dichlorofluorescein (DCF) diacetate as previously outlined [13]. Cells were treated with bosentan (10 μ M), BQ788 (10 μ M) or BQ123 (10 μ M) 30 min prior to the addition of 10% CSE and incubated for 24 h.

At the end of the incubation period, cells were washed twice with PBS and fluorescence was measured using a microplate spectrophotometer (Victor 1420; PerkinElmer, Madrid, Spain). Results were expressed as DCF fluorescence in relative fluorescence units. Representative images of each condition were captured *via* fluorescence microscopy.

Immunofluorescence

Precision-cut human lung slices were incubated in the presence or absence of bosentan, BQ788 or BQ123 for 1 h and stimulated with 10% CSE for 24 h. Then, lung slices were washed three times with PBS and fixed (4% paraformaldehyde

for 4 h at room temperature). Slices were embedded in OCT™ Compound (Tissue-Tek®, Alphen aan den Rijn, the Netherlands) and immunostained with ETA and ETB antibodies followed by the application of a secondary rhodamine-conjugated antibody. A comparative study of the autofluorescence of the internal and external elastic lamina enabled the distinction between endothelial and smooth muscle cells (SMCs) in small intrapulmonary arteries.

Preparation of precision-cut lung slices from resected human lung

Precision-cut lung slices were obtained as previously outlined [16] with minor modifications. Briefly, 3% (w/v) ultralow melting point agarose (Sigma, Poole, UK) was injected into the lung tissue, which was cut into precision-cut slices using a Krumdieck tissue slicer (model MD4000; Alabama Research and Development, Munford, AL, USA) with the slice thickness set at 260–300 μ m. We carefully chose only small arteries that were adjacent to identifiable small airways. Sections were placed in fresh medium and incubated in the presence or absence of bosentan (10 μ M), BQ788 (10 μ M) or BQ123 (10 μ M) for 1 h prior to stimulation with or without 10% CSE for 24 h. The slice was visualised using a microscope (Eclipse TE200, Nikon; magnification $\times 40$) connected to a live charge-coupled device camera (CoolSNAPfx; Photometrics, Tucson, AZ, USA). After washout (30 min), 80 mM KCl was perfused for 5 min to establish the maximal contractile response (100%). After rinsing and equilibration (normally 10 min of perfusion), the lowest concentration of ET-1 needed to begin the concentration response (10^{-9} – 10^{-6} M) was administered. Small pulmonary artery contraction was continuously monitored and was expressed as a percentage of the maximal reduction of area obtained with KCl. Artery lumen area was measured using MetaMorph software (Molecular Devices) and given in units of square micrometres. Results were expressed as % of KCl area. A log half-maximal effective concentration (EC₅₀) value and maximum drug effect (E_{max}) value for each artery was derived from a concentration–response curve. Details are given in the supplementary material online, in the Expanded Methods section.

Analysis of results

All values are reported as mean $\pm$ SEM. Determinations were performed in duplicate and at least three independent experiments were performed for each set of conditions. Two-group comparisons were analysed using the two-tailed paired t-test for dependent samples, or unpaired t-test for independent samples. Multiple comparisons were analysed by one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test. For all procedures, p-values of <0.05 were considered statistically significant.

RESULTS

CSE-induced ETB and ETA upregulation is prevented by bosentan

In vitro exposure of HPASMCs to CSE elicited a dose- and time-dependent increase of the ETA and ETB protein and mRNA expression (fig. 1a and b), reaching a peak value at 10% CSE after 24 h of stimulation. Based on these results, we selected 10% CSE for 24 h as the stimulation condition for later studies. Pre-incubation of HPASMCs with bosentan (10 nM to 10 μ M) dose-dependently prevented ETA and ETB protein

FIGURE 1. Cigarette smoke increases endothelin receptor A and B (ETA and ET_B, respectively). Cigarette smoke extract (CSE) a) dose- and b) time-dependently increased ET_B and ETA protein and mRNA expression in human pulmonary artery smooth muscle cells. c) Bosentan (BOS), d) BQ788 and e) BQ123 dose-dependently prevented the CSE-induced ET_B protein expression, while only BOS and BQ123 dose-dependently prevented ETA expression. f) BOS and BQ123 dose-dependently prevented the CSE-induced ET_B and ETA gene expression. g) BOS, BQ123 and BQ788 dose-dependently prevented the CSE-induced ETA expression. Data are presented as mean ± SEM of three independent experiments per condition. *: $p < 0.05$ compared with respective control (C) condition; #: $p < 0.05$ compared with CSE group.

upregulation (fig. 1c). The selective ET_B antagonist BQ788 prevented the CSE-induced ET_B upregulation at concentrations of 1 and 10 µM concentrations, while no effect was observed on CSE-induced ETA upregulation (fig. 1d). In contrast, the selective ETA antagonist BQ123 successfully prevented ETA upregulation at 100 nM to 10 µM and ET_B upregulation at 1 and 10 µM (fig. 1e). Studies performed on mRNA expression showed the same results as those observed for protein expression (fig. 1f and g).

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

Incubation of HPASMCs with the ERK1/2 inhibitor PD98059 (10 µM), the Rho-kinase inhibitor Y27632 (10 µM) or with the antioxidant *N*-acetylcysteine (NAC) (1 mM) effectively prevented ET_B and ETA protein overexpression induced by CSE (fig. 2a). Likewise, HPASMC incubation with a monoclonal antibody to ET-1 (10 µg·mL⁻¹) also suppressed CSE-induced ET_B and ETA overexpression (fig. 2a). In other experiments, ET-1 (10 nM) addition (24 h) increased ET_B and ETA protein expression (fig. 2b). Bosentan (10 µM), BQ123 (10 µM), PD98059, Y27632 or NAC partially prevented the ET-1-induced ET_B and ETA protein expression whilst BQ788 (10 µM) only prevented ET_B overexpression (fig. 2b). These results were in keeping with ET_B and ETA gene expression (fig. 2c). Interestingly, 10% CSE (24 h) significantly increased supernatant ET levels ($p < 0.05$ versus control; fig. 2d), which was prevented by bosentan, BQ788, BQ123, PD98059, Y27632 and NAC (1 mM) (fig. 2d).

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

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

Bosentan prevents CSE-induced HPASMC proliferation

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

HPASMC exposure to CSE increases the ET-1-induced [Ca²⁺]_i and cell contraction

HPASMCs exposed to 10% CSE were subject to an acute increase in 10 nM ET-1-induced [Ca²⁺]_i with a mean ± SEM peak value of 493 ± 46 versus 274 ± 34 nM for untreated cells (control) ($p < 0.05$; fig. 5a and b). Bosentan, BQ788 or BQ123 added before and during CSE exposure reduced the acute ET-1-induced peak increase in [Ca²⁺]_i to 304 ± 41, 395 ± 36 and 343 ± 32 nM, respectively ($p < 0.05$ versus CSE alone; fig. 5a and b). ET-1 (10 nM) increased cell contraction and was significantly higher in those cells exposed to CSE (10%) versus unexposed cells ($p < 0.05$; fig. 5c). Cells pre-treated with CSE in the presence of bosentan, BQ788 or BQ123 were less susceptible to ET-1-induced cell contraction, as their cell contraction was near to control levels (fig. 5c).

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

Precision-cut lung slices incubated with CSE showed an increase of ET_B protein expression, which was significantly prevented by bosentan, BQ788 and BQ123, while CSE-induced ETA protein expression was only prevented by bosentan and BQ123 (fig. 6a). These results were qualitatively reproduced by immunofluorescence experiments (fig. 6b). In this regard, CSE exposure increased the ET_B fluorescence intensity in endothelial cells and SMCs. ETA fluorescence intensity was augmented only in the area of SMCs. Precision-cut lung slices pre-treated with bosentan showed lower fluorescence intensity for both ET_B and ETA (fig. 6b). Precision-cut lung slices pre-treated with 10% CSE increased vascular sensitivity to ET-1. The calculated log EC₅₀ for the CSE treated group was -9.36 ± 0.32 versus -8.47 ± 0.16 M in controls ($p < 0.05$; fig. 6c and d). Precision-cut lung slices exposed to CSE in presence of bosentan, BQ788 or BQ123 significantly increased the ET-1 log EC₅₀ to -8.76 ± 0.3, -8.73 ± 0.17 and -8.64 ± 0.24 M, respectively. CSE also increased the ET-1 E_{max} contraction versus the control group (184 ± 5.7 versus 136 ± 3.6; $p < 0.05$; fig. 6f), which was attenuated by bosentan, BQ788 or BQ123 pre-treatments to an ET-1 E_{max} of 127.8 ± 4.3, 160.9 ± 3.6 and 145.8 ± 4.3, respectively (fig. 6f). However, pulmonary arteries from smokers and COPD patients showed an increase in ETA and ET_B protein expression versus nonsmoker patients ($p < 0.05$; fig. 7).

DISCUSSION

The relevance of this study is based in two main assumptions: 1) chronic CS exposure is responsible for pulmonary vascular remodelling and PH development during COPD progression [17]; and 2) the ET system is directly implicated in pulmonary remodelling in COPD patients [18]. The results of this study show that ETR antagonism could attenuate CS-induced HPASMC proliferation and tension, as well as small intrapulmonary artery tension, by means of ETR and ET down-regulation, thus suggesting that bosentan could be useful to treat certain forms of PH in smokers with COPD, as suggested recently [19, 20]. Furthermore, we provide a novel mechanism of action by which CS increases ETR expression through a feed-forward mechanism mediated by ET release and the activation of ERK1/2, RhoA-GTP and intracellular ROS. All these intracellular pathways were attenuated by bosentan.

FIGURE 2. Cigarette smoke increases endothelin receptor A and B (ETA and ETB, respectively) by a mechanism that implicates intracellular reactive oxygen species, extracellular signal-regulated kinase 1/2, RhoA-GTP and an autocrine endothelin (ET) feed-forward mechanism. a) The increase of ETA and ETB induced by 10% cigarette smoke extract (CSE) (24 h of incubation) was prevented by PD98059 (10 μ M), Y27632 (10 μ M) or *N*-acetylcysteine (NAC) (1 mM) in human pulmonary artery smooth muscle cells. Incubation with ET-1 monoclonal antibody (mAb) (10 μ g·mL⁻¹) 30 min before CSE addition also prevented ETA and ETB upregulation, unlike the negative immunoglobulin (Ig)G1 isotype control. Incubation with 10 nM ET-1 for 24 h significantly increased ETA and ETB b) protein and c) mRNA expression, which was prevented by bosentan (BOS) (10 μ M), BQ123 (10 μ M), PD98059, Y27632 and NAC, while BQ788 (10 μ M) only prevented ETB, but not ETA, expression. d) 10% CSE increased ET release, which was attenuated by BOS, BQ123, BQ788, PD98059, Y27632 and NAC. Data are presented as mean $\pm$ SEM of three independent experiments per condition. *: $p < 0.05$ compared with respective control condition; #: $p < 0.05$ compared with CSE group.

Whether our results are applicable to *in vivo* CS inhalation depends on the assumption that CS components reach the vascular bed of smaller pulmonary arteries. Several observations strengthen this assumption. 1) CS components are rapidly taken

up into the bloodstream during smoking (~1 min) [21], suggesting rapid equilibration across the gas-exchange surface. 2) Many smoke components are highly water soluble, such as peroxynitrite, which is considered a potent remodelling agent

FIGURE 3. Endothelin receptor antagonism attenuates the cigarette smoke extract (CSE)-induced reactive oxygen species (ROS), extracellular signal-regulated kinase (ERK)1/2 phosphorylation and RhoA-GTP activation. a, b) 10% CSE increases intracellular ROS generation after 24 h of exposure as assessed by dichlorofluorescein (DCF) fluorescence intensity in human pulmonary artery smooth muscle cells. The CSE-induced ROS generation was suppressed by bosentan (BOS) (10 μ M) and by BQ123 (10 μ M) but not by BQ788 (10 μ M). a) Representative intracellular ROS fluorescence images are presented. Scale bar=20 μ m. c) 10% CSE elicited ERK1/2 phosphorylation after 24 h of exposure, which was suppressed by BOS, attenuated by BQ123 and, to a lesser extent, by BQ788. d) 10% CSE increased RhoA-GTP activation which was also suppressed by BOS, attenuated by BQ123 and, to a lesser extent, by BQ788. Data are presented as mean $\pm$ SEM of three independent experiments per condition. RFU: relative fluorescence unit; pERK: phosphorylated ERK; OD₄₉₀: optical density measured at 490 nm. *: $p < 0.05$ compared with respective control condition; #: $p < 0.05$ compared with CSE group.

in pulmonary arteries [22], allowing easy solution into the alveolar lining and interstitial fluid. 3) Respiratory gas exchange has been demonstrated in pulmonary vessels as large as 3 mm [23], suggesting that smoke might have access to smaller pulmonary arteries; we obtained the same results in cells obtained from ~3-mm arteries as in small intrapulmonary arteries of 100 μ m internal diameter (precision-cut lung slice experiments). 4) Smoke components are present in recirculating blood for several hours after smoking [24],

allowing continued exposure to the lung vascular bed during recirculation.

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

FIGURE 4. Cigarette smoke increases pulmonary artery smooth muscle cell proliferation. a) 10% cigarette smoke extract (CSE) increased human pulmonary artery smooth muscle cell (HPASMC) proliferation after 24 h of exposure. Bosentan (BOS) (10 μ M), BQ123 (10 μ M), BQ788 (10 μ M), PD98059 (10 μ M), Y27632 (10 μ M), *N*-acetylcysteine (NAC) (1 mM) and endothelin (ET)-1 monoclonal antibody (mAb) (10 μ g·mL⁻¹) effectively prevented the CSE-induced HPASMC proliferation. b) platelet-derived growth factor (PDGF) at 10 ng·mL⁻¹ increased HPASMC proliferation by 3.1-fold. In the presence of CSE 10%, PDGF (10 ng·mL⁻¹) increased HPASMC proliferation 3.5-fold, which was attenuated by BOS (10 μ M), BQ123 (10 μ M) and BQ788 (10 μ M). Data are presented as mean $\pm$ SEM of three independent experiments per condition. Ig: immunoglobulin. *: $p < 0.05$ compared with respective control condition; #: $p < 0.05$ compared with CSE group; †: $p < 0.05$ compared with CSE+PDGF group.

The ET system appears to have a large impact on the initiation and progression of lung vascular remodelling. In fact, exhaled breath condensate and circulating ET-1 levels are increased in COPD patients with PH, and both are correlated with pulmonary systolic pressure [5]. Furthermore, animals exposed to CS show an increase in basal ET-1 levels and vascular contractility, which may contribute to the pulmonary pathophysiology associated with CS [26]. Recent studies

performed on rat arteries originating from various tissues, such as brain, mesentery and kidney, conclude that CSE upregulates ET β and ET α expression by a mechanism that implicates the activation or phosphorylation of ERK1/2, p38, JNK, protein kinase (PK)C and NF- κ B [9–11]. However, currently, no data about the effect of CS on ETR expression in human pulmonary arteries are available. In this study, we have observed for the first time that the CSE-stimulated

FIGURE 5. Cigarette smoke sensitises pulmonary artery smooth muscle cells to acute endothelin (ET)-1-induced intracellular calcium and cell contraction. Human pulmonary artery smooth muscle cells (HPASMCs) were exposed to 10% cigarette smoke extract (CSE) in the presence or absence of bosentan (BOS) (10 μ M), BQ123 (10 μ M) or BQ788 (10 μ M) for 24 h. a, b) Cells were washed three times with PBS and incubated with fura-2 acetoxymethyl ester for 30 min. Then, cells were stimulated with ET-1 (10 nM). Data are presented as nanomolar intracellular free calcium concentration ([Ca²⁺]_i) in 12 cells per experiment, in a total of four experiments per condition. c) Disks with cultured HPASMCs were placed in the microscope and cells imaged with bright-field illumination. The time course of contractile response of HPASMCs challenged with ET-1 (10 nM) is shown. Data are presented as total force exerted by the cell on the substrate in a total of six cells per condition. Data are presented as mean $\pm$ SEM. *: $p < 0.05$ versus control; #: $p < 0.05$ versus CSE.

FIGURE 6. Cigarette smoke increases endothelin receptor A and B (ETA and ETB, respectively) expression in human small intrapulmonary arteries, which increase pulmonary vascular contractility in response to acute endothelin (ET)-1. Small human intrapulmonary precision-cut slices were exposed to 10% cigarette smoke extract (CSE) in presence or absence of bosentan (BOS) (10 μ M), BQ123 (10 μ M) or BQ788 (10 μ M) for 24 h. a) Western blotting was performed to measure ETA and ETB protein expression. b) Precision-cut slices were fixed and immunofluorescence was performed for ETA and ETB with rhodamine secondary antibody. The internal elastic lamina (IEL) and external elastic lamina (EEL) were assessed by green autofluorescence. E: endothelial cells; M: smooth muscle cells. Scale bar=100 μ m. c) Representative images of the time-course human small pulmonary artery contraction in response to ET-1 under visible video microscopy. d–f) Concentration-dependent ET-1 contraction curves in two slices per condition of a total of three different patients per condition. Data are presented as mean $\pm$ SEM of a, b) three slices and d–f) two slices from three patients per condition. EC₅₀: half-maximal effective concentration; E_{max}: maximal drug effect. *: $p < 0.05$ versus control; #: $p < 0.05$ versus CSE.

increase in ETB and ETA protein and gene expression can be counteracted by the dual ETR antagonist bosentan as well as by the selective ETA antagonist BQ123. In contrast, the selective ETB antagonist BQ788 only prevented ETB overexpression.

This process was explained, in part, by a feed-forward mechanism mediated by ET release. In this regard, CSE was able to increase supernatant ET levels that were consequently suppressed by bosentan and BQ123 and, to a lesser extent, by

FIGURE 7. Endothelin receptor A and B (ETA and ETB, respectively) are upregulated in pulmonary arteries from smokers and chronic obstructive pulmonary disease (COPD) patients. Pulmonary arteries from nonsmokers, smokers and COPD patients were carefully isolated from tumour-free human lung tissue of surgical specimens. Total protein was extracted and probed for ETA and ETB antibodies by Western blot. Results represent the expression of ETA, ETB and the housekeeping protein β -actin from six nonsmokers, seven smokers and eight COPD patients. Densitometric data are shown as ETA and ETB protein expression relative to β -actin and normalised to nonsmoker expression. Data are presented as mean $\pm$ SEM of densitometry of one Western blot per patient. *: $p < 0.05$ versus nonsmokers.

BQ788. These results are apparently in contrast with previous reports where exposure to BQ788 and bosentan increased ET-1 mRNA in endothelial cells [27]. This effect was explained because extracellular levels of ET-1 are cleared *via* endocytosis of ETB; thus, its blockade impedes extracellular ET-1 clearance, inducing ET-1 upregulation. However, the results observed in this study were obtained in the totally different context of CSE exposure; CS increases ET release in endothelial cells and airway SMCs, as previously mentioned [28, 29]. In this study, we observed that the CS-induced ET release in HPASMCs was mediated by a mechanism including reactive oxygen generation, ERK1/2 phosphorylation and RhoA-GTP activation. This same mechanism has been also observed by our group in pulmonary artery endothelial cells [13]. Because blocking ETR is able to attenuate the CS-induced ROS, ERK1/2 phosphorylation and RhoA-GTP activation, it is reasonable to assume that ETR antagonism inhibits CS-induced ET release.

As with CSE, ET-1 incubation was also able to upregulate ETB and ETA expression in HPASMCs, which in turn confirms

similar results observed in HPAECs [13]. Since endothelial cells are the main reservoir of ET-1, one could hypothesise that CS increases ET-1 in endothelial cells, which could interact with HPASMCs, increasing ETR expression. So, a direct effect of ET-1 on CS-induced ETR overexpression in HPASMCs cannot be ruled.

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

It is known that intracellular ROS may activate several intracellular pathways, such as PKC, different MAPKs (*e.g.* ERK1/2, p38 and JNK) and transcription factors (*e.g.* NF- κ B) [30]. In fact, recent reports have shown that all these pathways are involved in CSE-induced ETB and ETA expression in rat basilar arteries [9, 10]. Furthermore, both CSE and ET-1 activate the reduced nicotinamide adenine dinucleotide phosphate oxidase complex to produce intracellular ROS [31, 32]. Interestingly, both the CSE oxygen species H_2O_2 and ET-1-induced intracellular ROS are mediated by the activation of ETA, since it has been shown that BQ123 inhibits them in fetal PSMCs [32]. In this study, the antioxidant NAC, bosentan and BQ123 attenuated the CSE-induced ROS, and ETB and ETA overexpression. In contrast, BQ788 did not influence the levels of ROS and ETA induced by CSE, so these results may explain, in part, the deficiency of BQ788 in affecting CSE-induced ETA expression. However, the ERK1/2 inhibitor PD98059 was sufficient in preventing the CSE and ET-1-induced ETB and ETA expression, which is in accord with previous reports in animal models [9, 11]. In this regard, bosentan, BQ123 and, to a lesser extent, BQ788 prevented ERK1/2 phosphorylation, thus highlighting the role of ERK1/2 in CSE-induced ETR upregulation.

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

During the course of pulmonary vascular remodelling, PSMC proliferation contributes to intimal thickening. Previously, studies have positively correlated CS to vascular SMC proliferation [34] by a mechanism involving ERK1/2 activation. In this study, we observed that bosentan, BQ123 and BQ788 prevented HPASMC proliferation in a process involving both forms of ETR. Coupled with this, HPASMC proliferation was mediated by the activation of intracellular ROS, ERK1/2 and Rho-kinase, as well as by the autocrine ET action.

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

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

Despite the novel mechanistic pathways studied here, we are aware of the study limitations. First, we conducted an *in vitro* acute (24 h) model of CS exposure, but progressive worsening of pulmonary haemodynamics in humans are induced only by chronic CS exposure over many years. Secondly, *in vitro* studies are not always representative of the *in vivo* findings, since a number of substances are released by CS and counter-act different cell types at the same time; thus, results observed in isolated HPASMCs could be different *in vivo*. Thirdly, although we have found ETR overexpression in pulmonary arteries from smokers and COPD patients, none of them showed PH, so whether COPD patients with PH have ETR upregulation or whether bosentan attenuates ETR upregulation *in vivo* remains unknown.

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

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STATEMENT OF INTEREST

None declared.

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