Supplementary materials

Bosentan inhibits cigarette smoke-induced ETA and ETB expression in small intrapulmonary arteries

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Expanded methods

Drugs and solutions

Unless stated otherwise, all reagents used were obtained from Sigma Chemical Co. (Madrid, Spain). Bosentan (provided by Actelion Pharmaceuticals Ltd), BQ788, BQ123, PD98059, N-acetyl-L-cysteine (NAC) and Y27632 were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock solutions. Several dilutions of the stocks were prepared, using cell culture medium. The final concentration of DMSO in the culture medium did not exceed 0.1% and had no significant pharmacological activity. At this respect we did not detected changes in basal ETA or ETB expression in HPASMCs during the culture (24 h and 48 h) conditions with or without vehicle (DMSO). ET-1 was dissolved in sterile water. Mouse monoclonal antibody against human ET-1 (mAb-ET-1) (Abcam, UK; cat. nº ab20940) was used at 10 μg/ml concentration to suppress the effect of the ET-1 supernatant levels as previously outlined [1]. Nonspecific mouse IgG1 was used at the same concentration as control. Every drug was incubated with cells for 30min, and 1h with human tissue before different stimulations.

Western blot

Western blot analysis was used to detect changes in ETB (50 kD), ETA (54 kD) and p-ERK1/2 (42-44 kD) as previously outlined [2, 3]. In brief, cells or precision cut-lung slices were scraped from a confluent 25-cm^2 flask or homogenate and lysed on ice with a standard lysis buffer. 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. Rabbit anti-human ETB (1:1,000) antibody (polyclonal antibody,1:1000, cat. nº sc-33537, Santacruz biotechnology, California) and rabbit anti-human ETA (1:1000, cat. nº E3651, Sigma) were normalized to mouse anti-

human β-actin (1:10,000) antibody (monoclonal antibody; Sigma; Catalogue no. A1978).). p-ERK1/2 expression was determined with the rabbit anti-human p-ERK1/2 (1:1,000) antibody (monoclonal antibody; Cell Signalling, USA; Catalogue no. 4376S) and was normalized to total rabbit anti-human ERK1/2 (1:1,000) antibody (monoclonal antibody; Cell Signalling, USA; Catalogue no. 4695). A peroxidase conjugated secondary (1:10,000) antibody with the enhanced chemiluminescence method ECL plus, (Amersham GE Healthcare, Buckinghamshire, UK) was used to detect labelled proteins. Densitometry of films was performed using the Image J 1.42q software (available at; http://rsb.info.nih.gov/ij/, USA). Results were expressed as ratios of the endogenous controls β-actin or total ERK1/2 as appropriate, and normalized to control group.

Preparation of precision-cut lung slices from resected human Lung.

Macroscopically normal human lung (3–10 g) was obtained from subjects undergoing lung resection as describe above. The tissue was weighed and then washed 3 times with 3 volumes of ice-cold slicing buffer (Hanks' balanced salt solution containing 100 units/ml penicillin and 0.1 mg/ml streptomycin). Immediately before preparation, the tissue was submerged in a reservoir containing ice-cold slicing buffer and 3% (w/v) ultralow melting point agarose (Sigma) injected into the tissue using a fine-gauge needle (NR18, Microlance; BD Biosciences, San Jose, CA). Injection aliquots (approximately 20 μl) were applied as evenly as possible throughout the tissue and were continued until the total agarose volume was 3 to 4 ml/g wet weight tissue. The inflated tissue was then left for a further 15 min at 0°C, at which point it was cut into small pieces in several different planes using a microtome blade. The cut faces of these slices were then inspected by eye for the presence of vascular or airway features. Cylindrical

cores of 8-mm diameter were then prepared containing these features oriented longitudinally along the core. These were further processed to precision-cut lung slices using a Krumdieck tissue slicer (model no. MD4000; Alabama Research and Development Munford, AL) with the slice thickness set on 260 to 300 μm. Slices were transferred, in order of preparation, to wells of a 12-well tissue culture plate containing 1 ml/well incubation buffer (RPMI 1640vcontaining 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 4vmM L-glutamine.). The culture plate was placed on a rocker arm in a humidified incubator (37°C, 5% CO2) for overnight incubation. The medium was changed at 1, 3, and 6 h, and after overnight incubation. This approach allows the agarose to dissolve out of the lung slice. To ensure that the explants were viable, airway epithelium was examined by light microscopy using an inverted microscope to detect beating cilia before the explant was utilized for vascular studies. The explants were then inspected to find vessels that had been sectioned in a transverse plane. We carefully chose only small arteries that were adjacent to identifiable small airways; the approximate internal diameter range of the untreated arteries was 200–400 μm.

Sections were placed in fresh media an incubated in presence or absence of bosentan, BQ788 or BQ123 for 1h prior to stimulation with or without CSE 10% for 24h. Two slices (n=2) per condition in a total of 3 different patients (p=3) were used. Each individual slice was place in a thermostated (37ºC) microscope chamber (Harvard apparatus, USA: cat. nº PH-6D) coupled to perfusion gas (5% CO_2) chamber (Harvard apparatus: cat. nº RC-29) and held in place using a platinum weight with nylon attachments. Slice was perfused with a multivalve perfusion system (Harvard apparatus: VC-66CS) and controlled with a valve flux (Harvard apparatus: Harvard apparatus: cat. nº FR-50) at 0.5ml/min. The slice was located using a microscope (Nikon Eclipse TE200; magnification, x40) connected to a live CCD camera CoolSNAPfx

photometrics. Slices were first washout with pefused Hanks' balanced salt solution for 30 min. A baseline live video imaging was recorded each 10 seconds. After washout, KCl 80mM was perfused during 5 min to establish the maximal contractile response (100%). After rinsing and equilibration (normally 10min of perfusion) the lowest concentration of ET-1 to begin the concentration response (10^{-9} to 10^{-6} M) was started. Each ET-1 concentration was perfused during 5 min (time in which area reached constant). Small pulmonary artery contraction was continuously monitored and was expressed as a percentage of the maximal reduction of area obtained with KCl. Areas were measured at the end and before the start of each ET-1 concentration. Artery lumen area was measured using a MetaMorph software (Molecular Devices, USA) and given in units of square micrometers. Results were expressed as % of KCl area. A log EC_{50} value and maximum drug effect (Emax) value for each artery was derived from a concentration-response curve.

Supplementary bibliography:

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