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Bosentan inhibits oxidative and nitrosative stress and rescues occlusive pulmonary hypertension

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Abstract

Pulmonary arterial hypertension (PH) is a fatal disease marked by excessive pulmonary vascular cell proliferation. Patients with idiopathic PH express endothelin-1 (ET-1) at high levels in their lungs. As the activation of both types of ET-1 receptor (ETA and ETB) leads to increased generation of superoxide and hydrogen peroxide, this may contribute to the severe oxidative stress found in PH patients. As a number of pathways may induce oxidative stress, the particular role of ET-1 remains unclear. The aim of this study was to determine whether inhibition of ET-1 signaling could reduce pulmonary oxidative stress and attenuate the progression of disease in rats with occlusive-angioproliferative PH induced by a single dose of SU5416 (200 mg/kg) and subsequent exposure to hypoxia for 21 days. Using this regimen, animals developed severe PH as evidenced by a progressive increase in right-ventricle (RV) peak systolic pressure (RVPSP), severe RV hypertrophy, and pulmonary endothelial and smooth muscle cell proliferation, resulting in plexiform vasculopathy. PH rats also had increased oxidative stress, correlating with endothelial nitric oxide synthase uncoupling and NADPH oxidase activation, leading to enhanced protein nitration and increases in markers of vascular remodeling. Treatment with the combined ET receptor antagonist bosentan (250 mg/kg/day; day 10 to 21) prevented further increase in RVPSP and RV hypertrophy, decreased ETA/ETB protein levels, reduced oxidative stress and protein nitration, and resulted in marked attenuation of pulmonary vascular cell proliferation. We conclude that inhibition of ET-1 signaling significantly attenuates the oxidative and nitrosative stress associated with PH and prevents its progression.

Keywords

Endothelin; Pulmonary hypertension; NADPH oxidase; Uncoupled eNOS; Free radicals

Severe pulmonary hypertension (PH) is a debilitating disease that occurs more frequently in women [1-3]. PH is characterized by a progressive increase in pulmonary artery pressure ultimately leading to right-heart failure and death. Although PH was initially considered to

be a disease of abnormal pulmonary vasoconstriction [4,5], vascular remodeling is now recognized as an important component in the pathogenesis of the disease [6,7]. PH is characterized by obliterative proliferation of apoptosis-resistant endothelial cells (ECs), medial hypertrophy, and the formation of complex vascular structures known as concentric and plexiform lesions [8-10].

Markers of increased oxidative stress have been detected in the lungs of patients with PH [11,12]. Oxidative stress is known to promote vasoconstriction, vascular remodeling, inflammation, and fibrosis [13]. These are all major pathological events present in patients with PH. Multiple enzymes and mechanisms may contribute to increased production of reactive oxygen species (ROS), including NADPH oxidase [14], xanthine oxidase [15], the mitochondrial electron transport chain [16], and uncoupled endothelial nitric oxide synthase (eNOS) [17,18] and decreased activity of antioxidant systems. The uncoupling of eNOS, i.e., switching enzymatic activity from NO to superoxide production, is of particular importance in PH, as eNOS uncoupling not only contributes to the oxidative stress itself, but also attenuates NO synthesis. Together with direct scavenging of NO by superoxide to limit NO bioavailability, this results in severe NO deficiency and endothelial dysfunction [19]. It has been previously shown that patients with PH have reduced amounts of NO in exhaled breath [20-23] and decreased amounts of stable NO metabolites (NO_x) in bronchoalveolar lavage fluid and urine [24,25]. Importantly, therapies that restore NO production are associated with improvements in pulmonary vascular resistance [21-23,25,26].

Endothelin-1 (ET-1) is a potent vasoconstrictor that also possesses strong mitogenic properties. Thus, ET-1 induces not only pulmonary vasoconstriction, but also the proliferation of pulmonary vascular smooth muscle cells [27-29]. ET-1 is highly expressed in the lungs of patients with PH, especially in the vessels most affected by morphological abnormalities [30]. The expression of ET-1 correlates with disease severity and outcome [31], suggesting an important role for ET-1 signaling in the pathobiology of PH. There are at least two types of endothelin receptor: ETA and ETB [32]. Activation of ETA receptors, located on vascular smooth muscle cells (SMCs), mediates the vasoconstrictive and mitogenic effects of ET-1. The role of ETB receptors is more complex, because ETBs expressed on SMCs can also promote vasoconstriction and cell proliferation, whereas those expressed on endothelial cells are responsible for ET-1 clearance, activation of NO, and prostacyclin release [33]. Previous studies have shown a significant upregulation of ETB receptor gene expression in patients with severe PH [34], suggesting that dual antagonism of ETA and ETB receptors may be an effective therapy for PH [35,36]. Thus, in this study we utilized the combined receptor antagonist bosentan to determine its effect on the progression of PH in a rat model of occlusive-angioproliferative PH induced by a single administration of the VEGF receptor 2 antagonist SU5416 followed by exposure to chronic hypoxia [37].

Materials and methods

Animals

A total of 32 female Sprague–Dawley rats (SD; 200–270 g) were used in this study ($n = 8$ per group). We used female rats, because in this model, similar to pulmonary arterial hypertension patients, females develop more severe disease than males [38]. Animals were obtained from Charles River (Wilmington, MA, USA) and were housed at a University of Pittsburgh animal care facility for at least 1 week before being used in this experiment. Animals were kept on a 12-h light/dark cycle at an ambient temperature of 22 °C and received standard rodent food and water ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and Georgia Health Sciences University.

Rat model of pulmonary hypertension

We utilized a rat model of occlusive-angioproliferative PH in which the disease develops after a single administration of the VEGF receptor 2 antagonist SU5416 (SU), followed by exposure to chronic hypoxia [37]. In this study we utilized the protocol established by Voelkel and Tuder and colleagues, which consists of high-dose SU5416 (200 mg/kg, sc) followed by 3 weeks exposure to hypoxia [37]. This protocol results in severe PH, accompanied by pronounced right atrial dilation and right-ventricular hypertrophy and irreversible occlusion of pulmonary arteries by endothelial cells. This model mimics many of the clinical and histopathological changes seen in human PH, i.e., occlusive remodeling of small pulmonary arteries with prominent media hypertrophy (“concentric lesions”), formation of complex glomeruloid-like vascular structures (“plexiform lesions”), and progressive disease character even after reexposure to normoxia. Before the study was begun, the rats were randomly allocated to one of four experimental groups: (1) control, SD rats receiving the vehicle for SU5416; (2) SU 10d, rats examined after a single injection of SU5416 (200 mg/kg) and 10-day exposure to hypoxia; (3) SU 21d, rats examined after a single injection of SU5416 and 21-day exposure to hypoxia; and (4) bosentan, rats subjected to a single injection of SU5416 and 21-day exposure to hypoxia and treated with bosentan (250 mg/kg mixed with rodent dough food) starting on day 10 of hypoxia. On day 0, animals assigned to chronic hypoxia (groups 2–4) were injected with a single dose of SU5416 (200 mg/kg sc) and placed into normobaric Plexiglas chambers (BioSpherix, Redfield, NJ, USA). The chambers were continuously flushed with nitrogen and oxygen ($10 \pm 0.5\%$) to maintain low CO₂ concentrations ($<0.5\%$) with O₂ and CO₂ concentrations continuously monitored (PROOX 110 BioSpherix oxygen controller and LB-2 CO₂ analyzer, Sensormedics).

Acute experiments

On day 10 and day 21 of exposure to hypoxia, subsets of animals were anesthetized (Inactin, 100 mg/kg ip) and instrumented for measurement of right-ventricle (RV) and systemic blood pressure. Briefly, a PE-240 polyethylene tube was inserted into the trachea to facilitate breathing. A customized pressure transducer catheter (SPR-513; Millar Instruments, Houston, TX, USA) connected to a Millar MPCU-200 pressure–volume conductance system was advanced into the right ventricle via the right jugular vein and right atrium. For systemic blood pressure measurement, the right carotid artery was cannulated with a fluid-filled PE-50 polyethylene catheter connected to a digital blood pressure analyzer (Micro-Med, Louisville, KY, USA). A 30-min stabilization period was permitted before a 30-min recording of right-ventricle pressure was conducted. At the end of pressure recording, the trachea catheter was connected to a Harvard rodent ventilator (Model 683; Harvard Apparatus, South Natick, MA, USA), the thorax was opened, and the lungs were flushed with saline (0.9% sodium chloride) via a catheter inserted into the right atrium to remove the blood from the pulmonary vessels. Animals were euthanized by anesthetic overdose, and the heart and lungs were dissected and weighed. The RV free wall was separated from the left ventricle and septum (LV + S) to determine the wet weights, the RV-to-body weight ratio (RV/BW), the LV + S-to-body weight ratio (LV + S/BW), and the RV-to-LV + S weight ratio (RV/LV + S). One lung was immersed in 10% buffered formalin for at least 72 h before being embedded, whereas the other was stored on -80°C and used for biochemical studies. Four-micrometer serial tissue sections from paraffin-embedded lungs were dewaxed and stained with hematoxylin and eosin (H&E) and Masson’s trichrome for histological and morphometric assessment.

Immunofluorescence

Paraffin sections (4 μm) were exposed to 55°C for 10 min, deparaffinized in xylene (3 $\times$, 5 min), and then hydrated using an alcohol series—100, 95, 70% alcohol (each 3 $\times$, 5 min)—and finally rinsed in water. The sections were processed for antigen retrieval by boiling the

slides in 10 mM citrate buffer (pH 6.0). The slides were cooled at room temperature for 20 min, washed in phosphate-buffered saline (PBS), and blocked in 10% normal serum overnight at 4 °C. Double-labeling immunofluorescence was then performed on serial sections from each group using rabbit anti-proliferating cell nuclear antigen (PCNA; Cell Signaling), goat anti-CD34 (Cayman Chemical Co.), mouse anti-eNOS (BD Transductions Labs, Lexington, KY, USA), and mouse anti-caldesmon (Sigma Chemical Co., St. Louis, MO, USA). The sections were incubated in primary antibodies for 1 h at room temperature. Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse antibodies (Molecular Probes) were then used to detect colocalization of PCNA with pulmonary endothelial cells (eNOS), proliferative endothelial cells (CD34), and pulmonary smooth muscle cells (caldesmon). In addition, CD34 was colocalized with eNOS. Sections were washed several times in PBS, mounted, and coverslipped in antifading aqueous mounting medium. Fluorescence-stained sections were then analyzed using the appropriate excitation and emission wavelengths as previously described [39] using a computer-based imaging system that consisted of an Olympus IX51 microscope equipped with a charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan). ImagePro Plus imaging software was used for acquisition of fluorescence images. For colocalization measurements in the pulmonary vessels, the fluorescence images were initially converted from RGB images to composite color images. The yellow channel generated from the overlay of the green and red fluorescence channels was then extracted utilizing ImageJ software (NIH). The resulting images were randomized and then the mean intensity and area of each vessel were determined by a blinded operator. The data were then reported as mean yellow intensity per vessel area.

Conventional immunohistochemistry

To further assess the cellular composition of the remodeled pulmonary vasculature of the animals, we immunohistochemically stained 4µm-thick formalin fixed paraffin imbedded slides for CD31 and smooth muscle actin (SMA), essentially as described [40]. The staining patterns/staining intensity were microscopically assessed in at least four different locations of the lung specimens. The use of tissue sections from humans with advanced pulmonary hypertension was approved by the Human Assurance Committee at the Hannover Medical School.

Western blot analysis

Whole lung tissues were homogenized in lysis buffer (1 M Tris, pH 7.4, 0.5% Triton X-100, 20% glycerol, 200 mM Na₃VO₄, 1 M NaF, 100 mM phenylmethanesulfonyl fluoride) plus a protease inhibitor cocktail (Sigma) and stored at -80 °C until used. Samples (100 µg of total protein) were incubated with 6 × sample buffer at 100 °C for 5 min and separated on 3–8% denaturing polyacrylamide gels (Invitrogen). All gels were then electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 1% bovine serum albumin in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 1 h and incubated overnight at 4 °C with an appropriate dilution of primary antibody. The antibodies used were anti-phospho Y1213-Flt-1 and anti-Flt-1 (both from Millipore), anti-phospho Y1059-VEGF receptor 2 and anti-VEGF receptor 2 (both from Cell Signaling), polyclonal anti-phospho-eNOS (Ser1177) (Millipore, Upstate), anti-eNOS and anti-inducible (i) NOS (BD Transduction Laboratories), and polyclonal anti-CD34 antibody (Santa Cruz Biotechnology) to estimate the levels of proliferating endothelial cells; monoclonal anti-caldesmon antibodies (Sigma) to estimate the levels of pulmonary SMCs; and monoclonal anti-PCNA antibody (Cayman Chemical Co.) and polyclonal anti-cyclin A (Millipore, Upstate) to estimate overall lung cellular proliferation. The antisera used to determine ETA and ETB proteins were prepared as previously described [41]. After being washed three times with TBS containing 0.1% Tween 20, membranes were incubated with the appropriate

secondary antibody conjugated to horseradish peroxidase (1:5000) for 1 h at room temperature. Membranes were washed three times with TBS containing 0.1% Tween 20 and protein bands were visualized using chemiluminescence procedures and the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). The band intensities were recorded using a Kodak ImageStation and analyzed using Kodak ImageStation software. Blots were reprobed with β -actin to normalize for protein loading. All samples were divided into four sets to measure the progression of the disease (control, SU 10d, and SU 21d groups) and the effect of bosentan treatment (control, SU 21d, and bosentan groups). During analysis each set was normalized to the control group. Further, some membranes were stripped and reprobed to image up to three different proteins (iNOS/caldesmon/ ETB; ETA/cyclin A; ETA/CD34; ETB/eNOS/CD34; eNOS/ETB). They were also reprobed for β -actin.

Immunoprecipitation

Tissue lysates (250 μ g) were incubated with 3 μ l anti-eNOS (BD Transduction Laboratories) or anti-manganese superoxide dismutase (MnSOD; antiserum prepared as previously described [42]) for 2 h at 4 °C. Protein G Plus/Protein A agarose beads (Calbiochem) were then incubated overnight at 4 °C. Immunoprecipitates were pelleted and washed four times with lysis buffer. Pellets were suspended in sample buffer, boiled for 6 min, and subjected to immunoblotting using an antibody specific for 3-NT residues (1:1000; Calbiochem). Blots were reprobed for either eNOS or MnSOD to normalize for immunoprecipitation efficiency. Representative bands were taken from different parts of the same membrane.

Dot-blot analysis

Ten micrograms of total lung protein (in 1 μ l) was spotted onto a nitrocellulose membrane (Bio-Rad). The membrane was then allowed to dry overnight at room temperature, blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 for 1 h, and incubated with antibodies specific for either 3-nitrotyrosine (3-NT; 1:1000; Calbiochem) or dityrosine (1:500; Northwest) for 1 h at room temperature. After being washed three times the membranes were incubated with the appropriate secondary antibody and analyzed as described above. Representative dots were taken from the different parts of the same membrane.

Measurement of superoxide levels in lung tissue

To detect superoxide generation in lung tissue, we performed electron paramagnetic resonance (EPR) measurements. Fresh-frozen tissues were powdered in liquid nitrogen to form a fine tissue powder. Four to six milligrams of tissue powder was then dissolved in 300 μ l of EPR buffer (PBS supplemented with 5 μ M diethyldithio-carbamate and 25 μ M desferrioxamine; Sigma–Aldrich) at 0 °C in a temperature-controlled cooling shaker (UltraCruz; Santa Cruz Biotechnology) to generate a suspension of tissue particles containing intact, not lysed, cells. We found that at 0 °C this tissue suspension does not generate a measurable amount of superoxide (not shown). The dissolved powder was then separated (75 μ l) between four 1.5-ml Eppendorf tubes containing the spin probe 1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (5 mg/ml) only, CMH and the NOS inhibitor ethylisothiourea (1 mM), CMH and the superoxide scavenger dihydroethidium (DHE; 5 μ M), or CMH and the NADPH inhibitor apocynin (10 μ M). Each tube was then incubated for 2 min at 0 °C. We utilized DHE to scavenge superoxide instead of PEG–SOD as we found that DHE exhibited higher cell permeability. Doses of PEG–SOD up to 200 U did not scavenge superoxide as efficiently as 5 μ M DHE (not shown). Thirty microliters of tissue suspension from each tube was then transferred to a glass capillary for 60 min at room temperature. Superoxide radical generation was then measured by reaction with the CMH spin probe at room temperature in a Magnettech M100 instrument

(Magnettech, Berlin, Germany). Amplitudes of the EPR spectra were analyzed using ANALYSIS 2.0 software (Magnettech). To quantitate the amount of superoxide per milligram of protein, we performed a standard reaction of the superoxide-generating enzyme xanthine oxidase in the presence of xanthine and CMH. A reaction curve was generated with 1 mU of xanthine oxidase in the presence of xanthine (1 mM in PBS, pH 7.4; Sigma) in EPR reaction buffer containing CMH. This generated 1 nM/min superoxide generation rate over a 1-h period at room temperature. Final superoxide generation in the tissue was then expressed as pmol/min/mg of tissue.

Estimation of peroxynitrite levels

To estimate the levels of peroxynitrite, peripheral lung tissues were pulverized. Then, each tissue (10 mg) was placed in a microfuge tube, 1 × PBS (100 µl) was added, and then the samples were vortexed three times for 10 s each. Tissue was then incubated with PEG-catalase (100 U) for 30 min (to inhibit hydrogen peroxide-dependent oxidation of dihydrorhodamine 123 (DHR123)) before being placed in a 96-well black plate in the presence of 5 µmol/L DHR123 in 1 × PBS for 1 h. The fluorescence of rhodamine 123 was measured using excitation at 485 nm and emission at 545 nm using a Fluoroskan Ascent microplate fluorimeter (Thermo Electron, Waltham, MA, USA). At the end of the experiment the protein concentration in the tissue was measured to normalize the fluorescence values for each sample.

In situ detection of hydrogen peroxide levels

A modified 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) oxidation method was used to detect H₂O₂ levels [43]. Briefly, snap-frozen lung tissue samples (20 mg) were powdered, and 100 µl of PBS was added to each tissue sample, vortexed (30 s), and centrifuged (14,000 rpm × 10 min) before incubation with H₂DCFDA (25 µM; Calbiochem) for 30 min in the dark in the presence or absence of catalase (100 U/ml; Sigma) to confirm that the oxidation of H₂DCFDA was H₂O₂ dependent. The samples were measured with excitation at 485 nm and emission at 530 nm in a Fluoroskan Ascent FL (Thermo Electron). The oxidation of H₂DCFDA using known concentrations of H₂O₂ was used to generate a standard curve and fluorescence units were then converted into µmol H₂O₂/min/mg protein.

Measurement of MnSOD activity

MnSOD activities were measured using a cytochrome *c*-based spectrophotometric assay [44]. To inhibit SOD1, samples were preincubated with 5 mM KCN solution. Samples (5 µl) were added to a 1:10 mixture of 50 µmol/L xanthine in 0.001 mol/L NaOH and 10 µmol/L cytochrome *c* in 0.1 mmol/L EDTA and 50 mmol/L phosphate buffer xanthine oxidase (0.18 mg) in 0.1 mmol/L EDTA, and 50 mmol/L phosphate buffer was then added and the absorbance at 550 nm was recorded for 3 min. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* oxidation by 50%.

Determination of nitric oxide metabolites

Nitrite content in plasma samples was determined using a chemiluminescence method previously reported by our laboratory [45]. Tissue samples were treated with cold ethanol for 1 h at −20 °C and then centrifuged at 20,000g to precipitate proteins that can interfere with nitric oxide measurements. Potassium iodide–acetic acid reagent was prepared fresh daily by dissolving 0.05 g of potassium iodide in 7 ml of acetic acid. This reagent was added to a septum-sealed purge vessel and bubbled with nitrogen gas. The gas stream was connected via a trap containing 1 N NaOH to a Sievers 280i nitric oxide analyzer (GE Analytical Instruments, Boulder, CO, USA). Samples were injected with a syringe through a silicone–Teflon septum. Results were analyzed by measuring the area under the curve of the

chemiluminescence signal using the Liquid software (GE Analytical Instruments). The resultant NO_x value represents NO and nitrite level per milligram of protein.

Measurement of lung tissue levels of endothelin-1

To evaluate the levels of ET-1 in rat lung tissue a modified protocol for peptide extraction from lung tissue was used (Phoenix Pharmaceuticals). Briefly, 100 mg of powdered lung tissue was lysed in 500 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, with protease inhibitor cocktail (Sigma)). After centrifugation (1600 g for 15 min at 4 °C) the supernatant was collected and stored at -80 °C. On the day of extraction a SEP-Pak C18 cartridge (Millipore) was equilibrated by sequential washing with 1 ml of buffer B (60% acetonitrile in 1% trifluoroacetic acid (TFA; Phoenix Pharmaceuticals)) and three washings with 3 ml of buffer A (1% TFA). The sample was then loaded and the peptide fraction was eluted with 3 ml of buffer B, evaporated to dryness, and stored at -20 °C. The amount of ET-1 in the peptide fraction was then analyzed using the Endothelin EIA kit (Enzo Life Sciences). On the day of measurement the samples were reconstituted with 250 μl of supplied assay buffer and immediately used for analysis according to the manufacturer's protocol. The amount of ET-1 measured was normalized on the total amount of peptides in the peptide fraction of each sample. For this purpose the peptide fraction was diluted 100 times with distilled H_2O and the total amount of peptides measured as previously described [46]. The mean optical density value was calculated and used to normalize the amount of peptide in each sample.

Statistical analysis

Results are presented as means $\pm$ SEM. Statistical analyses were performed using OriginPro 7.5. Group comparisons were carried out using one-factor nested (hierarchical) analysis of variance (1F-ANOVA). If ANOVA indicated a significant difference among the means, specific comparisons were made with a Fisher's LSD test.

Results

The injection of a single high dose of SU5416 (200 mg/kg) induced a significant reduction in the phosphorylation of VEGFR2 (Fig. 1A), but not VEGFR1 (Fig. 1B). These data suggest that SU5416 acts as a specific VEGFR2 antagonist. The combination of chronic hypoxia and SU5416 resulted in a progressive increase in pulmonary pressure and the development of severe PH in SD rats. At day 10 and day 21 of hypoxia, animals had a two- and threefold increase in RV peak systolic pressure (RVPSP), respectively, compared to healthy controls (Table 1). Treatment with high-dose bosentan (250 mg/kg), initiated on day 10, prevented further progression of the disease, as demonstrated by the fact that no further increases in RVPSP occurred (Table 1). The development of PH was accompanied by a significant increase in lung weight by day 10 (Fig. 2A), which further increased at day 21 of hypoxia. Bosentan treatment not only prevented this increase, but also reversed the already developed lung injury, i.e., the bosentan group examined at 21 days had reduced lung weight compared with the SU 10d group (Fig. 2A). Rats developed severe RV hypertrophy and presented a nearly threefold increase in RV/BW ratio compared to controls (Fig. 2B). Moreover, after 21 days of hypoxia they developed left-ventricle hypertrophy (Fig. 2C), most probably due to the elevated systemic blood pressure (Table 1). The development of left-ventricle hypertrophy resulted in an underestimation of the Fulton index (Fig. 2D), and RV/LV + S ratio increased by only 224% relative to the control group, whereas RV/body weight ratio increased by 293% relative to the control group. Importantly, treatment with bosentan prevented further hypertrophy of the right and left ventricles beyond that observed at day 10 of hypoxia (Figs. 2B–D).

Compared to control rats, pulmonary hypertensive animals also developed an occlusive vasculopathy characterized by concentric lesions with marked medial hypertrophy and concentric intimal fibrosis (Figs. 3A and B). These histopathological changes correspond to those seen in humans with PH and vascular remodeling, recapitulating a Heath and Edwards grading of 3 and upward ("concentric lesions"; Figs. 3C and D). On average, the media of these concentric lesions measured 15% of the vascular diameter, whereas the remodeled intima made up ~60% of the total diameter of the artery. On average 70% of the total cells/nuclei of the vascular wall were located in the media and about one-third in the remodeled intima. Animals in the SU + hypoxia group exhibited concentric lesions, as well as circumscribed glomeruloid-like vascular formations, originating from the small pulmonary arteries (Figs. 3C–E). These structures were made up of slender, CD31-positive channels containing some luminal erythrocytes and were backed by continuous SMA-positive myogenic layers (Figs. 3K–M). These changes mimicked those seen in plexiform lesions in human patients with severe pulmonary hypertension (Fig. 3F) [40,47,48]. In contrast, hypertensive animals treated with bosentan had less severe vascular remodeling that was limited to medial hypertrophy with no plexiform lesions being observed (Figs. 3G–J).

The development of PH was also associated with a significant increase in the levels of ET-1 (Fig. 4) and both ETA (Fig. 5A) and ETB receptors (Fig. 5B). Bosentan attenuated the increase in ET-1 (Fig. 4). Bosentan also attenuated the increases in ETA (Fig. 5A) and ETB (Fig. 5B) protein levels. It is well documented that ET-1 signaling promotes oxidative stress induction through increased formation of both superoxide and hydrogen peroxide [29,43,49–52] and we found that superoxide levels were markedly increased in the lungs in PH rats (Fig. 6A). Both NADPH oxidase activation and eNOS uncoupling contributed to the enhanced levels of superoxide (Fig. 6A). In addition, PH produced a significant increase in H₂O₂ levels (Fig. 6B) and protein dityrosine formation levels (a stable marker of H₂O₂ generation, Fig. 6C), whereas bosentan prevented the increase in both superoxide and H₂O₂ (Figs. 6A–C).

The development of PH produced a progressive increase in eNOS protein levels that correlated with EC proliferation (Fig. 7). However, when eNOS levels were normalized by the prevalence of CD34-positive cells, as a marker of EC number, there were no significant differences between control animals and animals with PH (Fig. 7). These uniform eNOS/CD34 ratios suggest that the increase in eNOS protein was predominantly due to increased proliferation of pulmonary EC. Bosentan treatment reduced both CD34 and eNOS protein levels (Fig. 7). Our results also show that PH induced a progressive increase in the levels of iNOS (Fig. 8A), whereas bosentan treatment reversed iNOS levels to control values (Fig. 8A). Further, we found that the lungs from rats with occlusive PH had increased levels of peroxynitrite (Fig. 8B) and total protein nitration, one of the main consequences of peroxynitrite-mediated reactions (Fig. 8C), whereas bosentan treatment prevented the formation of peroxynitrite and the accumulation of nitrated proteins (Figs. 8B and C).

Nitration of protein tyrosine residues is a stable posttranslational protein modification commonly leading to inhibition of protein activity. Among the proteins known to be nitration-inhibited are MnSOD and eNOS [53–55]. Our results show that nitration of both MnSOD (Fig. 9A) and eNOS (Fig. 10A) was markedly increased in PH rats and that this nitration was accompanied by significant reductions in MnSOD activity (Fig. 9B), eNOS Ser117 phosphorylation (Fig. 10B), and NO bioavailability (Fig. 10C). Treatment with bosentan reduced the nitration of both MnSOD (Fig. 9A) and eNOS (Fig. 10A). This effect was accompanied by restoration of MnSOD activity (Fig. 9B) and increased levels of eNOS Ser117 phosphorylation (Fig. 10B). NO bioavailability was also increased by bosentan treatment (Fig. 10C). These data agree with clinical studies showing increased levels of

exhaled NO and whole-body NO production in patients with idiopathic PH after treatment with bosentan [21].

To examine the effect of bosentan on pulmonary vascular remodeling, we examined the expression of CD34 and caldesmon, selective markers of EC and SMC differentiation/proliferation in arteries, respectively. We also examined the expression of nonselective markers of cell proliferation PCNA and cyclin A. On day 21, animals with occlusive PH had significantly increased expression of CD34 (Fig. 7), caldesmon (Fig. 11), PCNA (Fig. 12A), and cyclin A (Fig. 12B). Bosentan treatment reduced expression of all four of these cell proliferation markers (Figs. 7, 11, and 12).

Because whole lung tissue was used for Western blot analysis, we performed additional immunohistochemical studies to localize the origin of the proliferating cells and to quantify the colocalization of the proliferative marker, PCNA, with both endothelial and smooth muscle cells. There was a relatively low amount of PCNA-positive cells in the small pulmonary arteries of control animals (Figs. 13A–E). However, the development of PH was associated with an increase in the level of PCNA staining, which by 21 days showed a high colocalization with caldesmon (Figs. 13A and E), eNOS (Figs. 13B and E), and CD34 (Figs. 13C and E). The progression of the disease was also associated with an increased number of CD34-positive cells (Fig. 13C) that colocalized with eNOS-positive cells (Figs. 13D and E). Bosentan treatment reduced the signals from PCNA, caldesmon, eNOS, and CD34 (Figs. 13A–E).

Discussion

It is well documented that ET-1 signaling is upregulated in both animal models and patients with PH. Thus, endothelin receptor inhibition should produce beneficial effects in these patients. During the past decade, a number of ET receptor antagonists have been developed and tested. These are classified into two categories, selective and nonselective. Initial efforts have focused on ETA selective antagonism, because ETB located on ECs is also known to stimulate NO-mediated vasodilation [56,57] and to participate in endothelin clearance [58,59]. However, more recent studies using isolated human small pulmonary arteries have suggested that a combined blockade of both ETA and the ETB is necessary to achieve optimal inhibition of vasoconstriction [60–62]. This may be due to the fact that activation of smooth muscle ETB receptors contributes to the proliferation of pulmonary SMCs [28] coupled with the fact that increased expression of the ETB receptor has been found in the pulmonary arteries of patients with severe PH [34]. Together these findings suggest that ETB receptors located on SMCs may be involved in the development of PH and, thus, targeting both ETA and ETB receptors may be more effective than selective antagonism of ETA receptors [63–66].

In this study we found that rats with occlusive PH have significantly increased expression of ET-1 and both types of ET receptors. The particular localization of the ETB receptors was not investigated, although the fact that bosentan significantly increased NO bioavailability suggests that the increased ETB protein amount was attributable mostly to the smooth muscle ETB receptor subtype, as previously shown in humans [34]. In general, the increased production of an agonist results in the downregulation of its receptor. However, in a disease state this compensatory mechanism may be overcome and facilitate the severity of disease. Indeed, there is previously published evidence of enhanced ET-1 and endothelin receptor expression in chronic hypoxia [67] and in pulmonary hypertensive patients [28,34,68]. Therefore, the occlusive model of PH seems to be a model that reproduces the main pathological changes in lungs of human patients with PH. The particular mechanisms and possible factors and stimuli for the upregulation of ET-1 and its receptors are poorly

understood. Our study was not designed to investigate these factors, although we can speculate that under hypoxic conditions increased expression of ETA and ETB could be achieved, for instance, through the HIF-1-mediated pathway. HIF-1 is known to induce the expression of both ET-1 and ET receptors [69,70]. ET-1 signaling, in turn, stabilizes HIF-1 [71], which could create a continued positive feedback loop and result in the uncontrolled vascular cell proliferation observed in this model. However, this vicious circle can be effectively interrupted by ET receptor antagonism.

Despite a number of animal and clinical studies reporting the benefits of ET receptor antagonism for the treatment of PH, it still remains unclear whether upregulation of ET-1 signaling is one of the primary reasons of PH development or a just a secondary event contributing to the severity of disease [72]. The previously reported efficacy of ET receptor antagonism in reducing hypoxic PH highlights the importance of ET-1 signaling in the pathogenesis of PH [72]. However, chronic hypoxia exposure produces only a mild and reversible form of PH, which does not represent the debilitating PH commonly seen in humans. The data we present provide evidence of a critical role for ET-1 signaling in the progression of the severe occlusive “human-like” form of PH. Indeed, the model we utilize, in which VEGF receptor antagonism is coupled with chronic hypoxia, is the only model among those currently in use that leads to the formation of concentric and plexiform lesions in pulmonary arteries [73]. In this study we utilized the previously published protocol using a higher concentration of SU5416 [37] over a relatively short duration (3 weeks) and were able to confirm the markedly increased endothelial proliferations that partially or completely occluded the lumen of pulmonary arteries, and formation of glomeruloid-like vascular proliferations, mimicking characteristic changes in plexiform vasculopathy seen in humans with severe pulmonary hypertension [47,48] and in rats treated with a lower dose of Sugen but examined after 12–14 weeks [73]. Importantly, inhibition of ET-1 signaling in this model was enough to completely abolish the progression of the disease by preserving RVPSP, RV, LV, and lung hypertrophy; attenuating lung EC and SMC cell proliferation; and preventing the formation of plexiform lesions.

Although the ability of ET-1 to induce ROS production in cell culture studies is well established, the role of ET-1 signaling in the oxidative stress associated with the development of PH is less well understood. Our data indicate that severe oxidative stress develops as early as day 10 after the insult, which correlates with early increases in both ETA and ETB expression. This fact, together with the ability of bosentan to attenuate the development of oxidative stress, indicates that the oxidative stress in this occlusive-angioproliferative model of PH is ET-1 dependent. Increased levels of superoxide are known to be responsible for significant reduction of NO bioavailability because of its ability to directly scavenge NO. This scavenging not only compromises the vasodilatory, antiproliferative, antithrombotic, and anti-inflammatory properties of NO, but also results in the formation of highly deleterious peroxynitrite that, in turn, induces nitration of cell proteins. Many proteins are known to be susceptible to nitration and in particular the nitration of MnSOD and eNOS inhibits their activity [53–55]. MnSOD is among the most important enzymes in the first line of oxidative stress defense, and its inhibition would inevitably exacerbate the severity of further oxidative stress. Nitration of eNOS is capable of inducing eNOS uncoupling that, again, significantly contributes to oxidative stress [54]. In our model, the development of PH is associated with a significant increase in levels of peroxynitrite and total protein nitration, as well as increased nitration of MnSOD and eNOS. As expected, nitration of MnSOD resulted in a significant reduction in MnSOD activity, whereas nitration of eNOS may be responsible for the decreased levels of eNOS phosphorylation and NO generation observed in rats with PH. It is likely that the loss of activity of these two enzymes enhances the severity of oxidative/nitrosative stress. Importantly, the reversibility of these pathological changes with bosentan with both

oxidative and nitrosative stress returning to control values suggests that oxidative/nitrosative stress may be the primary target of ET signaling in PH.

The reduction in NO bioavailability, due both to inhibition of eNOS and to direct NO scavenging by superoxide, will inevitably compromise its antiproliferative activity. Together with ROS-mediated mitogenic stimulation, the reduction in NO bioavailability is probably responsible for the increased vascular cell proliferation we observed in rats with severe PH. The markers of proliferation of both pulmonary ECs and SMCs were significantly increased on day 21 of PH, although the extents of the proliferation were different, with CD34-positive ECs showing a fourfold increase relative to controls, versus a threefold increase in caldesmon-positive SMCs. The same pattern was revealed by immunohistochemical analysis, with colocalization of PCNA and CD34 or eNOS found earlier (at day 10) compared to caldesmon. This outcome is in accordance with previous studies showing that SU5416 causes initial EC apoptosis with subsequent uncontrolled proliferation of the surviving apoptosis-resistant ECs, and SMC proliferation occurring as a secondary event [74]. The initial CD34-positive ECs are then capable of transdifferentiation into SMC-like cells and directly stimulating further SMC growth, inducing the secondary media thickening of pulmonary arteries. Importantly, bosentan treatment significantly reduced proliferation of both cell types to near-control levels, indicating that ET signaling plays a critical role in the uncontrolled proliferation of pulmonary vascular cells in PH.

One limitation of our study is that we have not directly elucidated the mechanism for the protective role of bosentan. It is possible its efficacy could be due, at least in part, to a reversal of the vasoconstriction that occurs early in the development of PH. However, the RVPSP in bosentan-treated rats (at day 21) is not significantly lower than in animals at day 10, when bosentan treatment was initiated, which suggests that bosentan is not an active vasodilator. This conclusion is also supported by previously published studies. For example, bosentan given at the early stages of disease (FCII of World Health Organization classification) for 6 months significantly increased the mean 6-min walk distance, but induced only modest changes in PAP [75]. A similar significant improvement in 6-min walk distance and improvement in WHO functional class, but mild reduction in PAP, was shown in a study in which bosentan treatment started at a late stage of PH (FCIII and FCIV) [76]. Importantly, bosentan treatment prevents the proliferation of pulmonary endothelial and smooth muscle cells (Figs. 7, 11, 13A, and 13B), reducing total proliferative signaling in the lung (Figs. 12 and 13). This suggests that bosentan, rather than acting as a vasodilator, has an antiproliferative effect. This may be due to a restoration of NO signaling, as eNOS is already uncoupled by day 10 (Fig. 6A). With disease progression further increases in eNOS protein levels are observed. However, as it is uncoupled, there is not an equivalent increase in NO bioavailability. Therefore, the animals with PH have pronounced endothelial dysfunction despite having increased eNOS protein levels. It has previously been shown that ET-1-mediated activation of NADPH oxidase leads to eNOS uncoupling and further increases in superoxide production [50], and in our model we found that both processes—NADPH oxidase activation and eNOS uncoupling—contribute to the increased oxidative stress. The reasons for the eNOS uncoupling are unclear but may be due to the oxidation of the eNOS cofactor tetrahydrobiopterin [77,78], or simply due to eNOS nitration [54]. Indeed, the observed increase in eNOS uncoupling correlates with increased eNOS nitration. Notably, this positive feedback loop of constant oxidative stress was ET signaling dependent, because bosentan treatment reduced superoxide formation to almost control levels. Thus, ET signaling seems to be responsible for initiation of the oxidative and nitrosative stress that, in turn, may result in the subsequent pathological events observed in the development and progression of PH, including endothelial dysfunction, decreased NO bioavailability, and uncontrolled vascular cell proliferation.

The important role of oxidative stress in PH has been previously established because antioxidant treatment or SOD overexpression ameliorates PH and restores the vasodilatory capability of pulmonary arteries [79,80]. We add to these findings by demonstrating that PH-mediated upregulation of ET receptor signaling induces self-perpetuating oxidative and nitrosative stresses that ultimately lead to the development of PH. We base this conclusion on the finding that nonselective inhibition of ET signaling is sufficient to completely abolish the oxidative and nitrosative stress and prevent the progression of PH. However, it is worth noting that bosentan treatment did not fully reverse the PH phenotype. Rather the treatment with bosentan seems to only prevent the progression of the disease. Thus, we speculate that our treatment duration may not be optimal to reverse the disease and that longer exposure times may be required. In support of this it has been shown that the complete reduction in medial thickness induced by the relatively mild stimulus of chronic hypoxia takes 6 weeks after the animals are returned to normoxia [81]. Therefore, in conclusion, we speculate that the administration of bosentan for a longer period could provide greater beneficial results, perhaps leading to the reversal of the PH phenotype.

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Fig. 1. SU5416 attenuates the activity of VEGFR2 but not VEGFR1. The activities of VEGFR1 and -2 were determined by Western blot analysis in peripheral lung tissue of control and PH rats. The levels of (A) pY1059 in VEGFR2 and (B) pY1213 in VEGFR1 were determined as well as the levels of total VEGFR2 and VEGFR1. At both 10 and 21 days after SU5416 injection the level of normalized p-VEGFR2 (A) was significantly attenuated, whereas the level of p-VEGFR1 (B) was unchanged. There was a high variability in total VEGFR1 protein levels; thus a representative image containing $n = 2$ is shown. Results are expressed as means $\pm$ SEM; $n = 6$ for VEGFR2 and $n = 5$ for VEGFR1. * $P < 0.05$ vs control group.

Fig. 2.

Bosentan treatment attenuates the development of RV hypertrophy and lung injury in rats with occlusive-angioproliferative pulmonary hypertension. (A) Lung, (B) right ventricle, and (C) left ventricle plus septum wet weight normalized to body weight were determined. In addition, (D) the Fulton index was measured. The development of PH was associated with lung and right- and left-ventricle hypertrophy. Development of LV hypertrophy resulted in underestimation of the Fulton index. Bosentan treatment prevented PH progression. Results are expressed as means $\pm$ SEM; $n = 8$ in each group. * $P < 0.05$ vs control group; † $P < 0.05$ vs SU 21d group.

Fig. 3. Histological changes in the lungs of rats with occlusive-angioproliferative pulmonary hypertension. Vascular changes were investigated (C–F) in SU 21d rats and (G–J) in rats with pulmonary hypertension treated with bosentan compared to (A and B) controls. Pulmonary hypertensive animals developed occlusive vasculopathy with prominent media hypertrophy and endothelial proliferation (C and D). Also, plexiform lesions, complex vascular formations that sprouted from small pulmonary arteries, were detected in the SU 21d group (E; arrow, plexiform lesion; black star, pulmonary artery; white star, bronchus). These mimicked plexiform lesions found in human patients with severe pulmonary arterial hypertension (F; arrow, plexiform lesion in human). As in humans, the changes in the PH rats were made up of CD31-positive endothelial channels, continuously backed by a homogeneous interstitium made up of SMA-positive spindle cells and branched from pulmonary arteries ((K) HE staining; (L) SMA staining of the myogenic interstitial cells; (M) CD31-positive endothelial cells; the adjacent pulmonary artery is indicated by *). Bosentan-treated animals showed less severe vascular remodeling limited to medial hypertrophy, with no glomeruloid-like lesions being observed in this group (G–J). (A), (C),

(G), (I) H&E; (B), (D), (H), (J) Verhoff-Van Gieson, original magnification 100–400 ×. Images are representative of six animals evaluated in each group and changes seen in patients with severe pulmonary arterial hypertension.

Fig. 4. Bosentan treatment attenuates the increase in endothelin-1 levels in the lungs of rats with occlusive-angioproliferative pulmonary hypertension. A peptide fraction was extracted from peripheral lung tissue and used to measure the levels of ET-1 by ELISA. ET-1 levels are significantly increased at 21 days in the PH rats and this is prevented by bosentan treatment. Results are expressed as means $\pm$ SEM (control $n = 4$, SU 10d $n = 4$, SU 21d $n = 3$, Bosentan $n = 5$). * $P < 0.05$ vs control group; † $P < 0.05$ vs SU 21d group.

Fig. 5.

The increase in ET receptor protein levels in rats with occlusive-angioproliferative pulmonary hypertension is attenuated by bosentan treatment. Protein levels of (A) ETA and (B) ETB subtype receptors were determined by Western blot analysis in peripheral lung tissue of rats with PH. Representative images showing the progression of PH (control, SU 10d, SU 21d groups) and treatment effect (control, SU 21d, bosentan groups) are presented for each. Densitometric analysis of the bands was carried out and normalized to control. Both ETA and ETB receptor expression is increased during the development of PH and this is attenuated by bosentan treatment. Results are expressed as means $\pm$ SEM ($n = 5$ for ETB SU 21d, $n = 6$ for all other groups). * $P < 0.05$ vs control group; † $P < 0.05$ vs SU 21d group.

Fig. 6.

Bosentan treatment attenuates the oxidative stress in lungs of animals with occlusive-angioproliferative pulmonary hypertension. The levels of superoxide in lung tissues were measured by EPR. (A) The development of PH is associated with an increase in superoxide generation as early as day 10. The primary sources of the superoxide are NADPH oxidase and eNOS. Bosentan prevents the increase in superoxide. (B) H₂O₂ levels were measured by oxidation of H₂DCFDA in the presence or absence of catalase. Bosentan treatment prevents the increase in H₂O₂ levels. (C) Total levels of tissue dityrosine were also determined by dot-blot analysis. A representative image is shown. Densitometric analysis indicates that dityrosine levels are increased at 21 days of PH and this is blocked by bosentan. Results are expressed as means $\pm$ SEM; (A) $n = 4$; (B) Control $n = 8$, SU 10d $n = 8$, SU 21d $n = 7$, bosentan $n = 8$; (C) control $n = 6$, SU 10d $n = 7$, SU 21d $n = 8$, bosentan $n = 6$. (A) $*P < 0.05$ vs control group; $\dagger P < 0.05$ vs SU 10d group; $\ddagger P < 0.05$ vs SU 21 group. (B, C) $*P < 0.05$ vs control group; $\dagger P < 0.05$ vs SU 21d group.

Fig. 7.

Expression of eNOS and CD34 in rats with occlusive-angioproliferative pulmonary hypertension. (A) Total eNOS and CD34 (a marker of endothelial cell number) were determined by Western blot analysis in peripheral lung tissue of rats with PH. Representative images showing the progression of PH (control, SU 10d, SU 21d groups) and treatment effect (control, SU 21d, bosentan groups) are presented for each. (B) Densitometric analysis of the bands was carried out and normalized to control. The significant increase in eNOS protein levels observed when normalized to β -actin (A) is lost when normalized to CD34 (B). Bosentan treatment normalized eNOS protein levels (A). Results are expressed as means $\pm$ SEM; $n = 5$ SU 21d, $n = 6$ for all other groups. * $P < 0.05$ vs control group; † $P < 0.05$ vs SU 21d group.

Fig. 8.

Expression of iNOS and markers of nitrosative stress in rats with occlusive-angioproliferative pulmonary hypertension. (A) iNOS protein levels were determined by Western blot analysis in peripheral lung tissue of rats with PH. Representative images showing the progression of PH (control, SU 10d, SU 21d groups) and treatment effect (control, SU 21d, bosentan groups) are presented. (B) The formation of peroxynitrite in peripheral lung tissue was estimated by measuring the levels of DHR123 oxidation in the presence of PEG-catalase. (C) Total protein nitration was determined by dot-blot analysis using an anti-3-NT antibody. A representative image is shown. PH correlates with an increase in iNOS protein levels (A), peroxynitrite formation (B), and total protein nitration (C). Bosentan treatment prevents these increases (A–C). Results are expressed as means $\pm$ SEM. (A) $n = 6$; (B) control $n = 7$, SU 10d $n = 8$, SU 21d $n = 7$, bosentan $n = 6$; (C) control $n = 5$, SU 10d $n = 7$, SU 21d $n = 7$, bosentan $n = 6$. * $P < 0.05$ vs control group; † $P < 0.05$ vs SU 21d group.

Fig. 9.

Bosentan treatment attenuates both the nitration and the inhibition of MnSOD in rats with occlusive-angioproliferative pulmonary hypertension. (A) Nitration of MnSOD in lung tissue was analyzed by immunoprecipitation. A representative image is shown.

Densitometric analysis of the bands was also carried out indicating that PH causes an increase in the nitration of MnSOD. (B) Further, this increase in nitration correlates with a decrease in MnSOD activity. Bosentan treatment prevents the increase in MnSOD nitration (A) and preserves its activity (B). Results are expressed as means $\pm$ SEM; (A) $n = 4$; (B) $n = 6$. * $P < 0.05$ vs control group; † $P < 0.05$ vs SU 21d group.

Fig. 10.

Bosentan treatment prevents the nitration of eNOS and preserves NOS activity and NO bioavailability in rats with occlusive-angioproliferative pulmonary hypertension. (A) Nitration of eNOS and (B) phosphorylation of eNOS at Ser1177 in lung tissue were analyzed by immunoprecipitation followed by Western blot analysis. Representative images are shown. Densitometric analysis of the bands indicated that PH increases the nitration of eNOS and this is prevented by bosentan treatment (A). Further, (B) the decrease in phosphorylation of eNOS at Ser1177, as well as (C) the decrease in plasma NO_x levels, is also prevented by bosentan. Results are expressed as means $\pm$ SEM; (A) $n = 4$; (B) $n = 4$; (C) $n = 5$ for SU 10d; $n = 6$ for all other groups. * $P < 0.05$ vs control group; † $P < 0.05$ vs SU 21d group.

Fig. 11.

Bosentan attenuates the proliferation of pulmonary vascular smooth muscle cells in rats with occlusive-angioproliferative pulmonary hypertension. Caldesmon protein levels were determined by Western blot analysis in peripheral lung tissue of rats with PH.

Representative images showing the progression of PH (control, SU 10d, SU 21d groups) and treatment effect (control, SU 21d, bosentan groups) are presented for each. Densitometric analysis of the bands was carried out and normalized to control. Results are expressed as means $\pm$ SEM; $n = 5$ for SU 21d, $n = 6$ for all other groups. * $P < 0.05$ vs control group; † $P < 0.05$ vs SU 21d group.

Fig. 12.

Bosentan attenuates the expression of proliferative markers in lungs of rats with occlusive-angioproliferative pulmonary hypertension. Pulmonary cell proliferation was estimated using Western blot analysis to quantify the levels of the proliferation markers (A) PCNA and (B) cyclin A. Representative images showing the progression of PH (control, SU 10d, SU 21d groups) and treatment effect (control, SU 21d, bosentan groups) are presented for each. Densitometric analysis of the bands was carried out and normalized on control. Results are expressed as means $\pm$ SEM; (A) $n = 6$; (B) $n = 5$ for control, $n = 6$ for all other groups. * $P < 0.05$ vs control group; † $P < 0.05$ vs SU 21d group.

Fig. 13.

Localization of PCNA with caldesmon, eNOS, and CD34 in small pulmonary arteries of rats with occlusive-angioproliferative pulmonary hypertension. To identify which cell types in the pulmonary arteries are proliferating, lung sections were costained with the proliferative marker PCNA and markers of endothelial (eNOS and CD34) and smooth muscle (caldesmon) cells. The representative images consist of each individual staining, costaining, and colocalization (yellow channel). To quantify the level of colocalization, the mean yellow intensity per vessel area was measured. The development of PH induces an increase in PCNA-positive cells in all layers of the small pulmonary arteries. (A and E) Colocalization of PCNA staining with the smooth muscle cell marker caldesmon is most pronounced in the SU 21d group. Bosentan treatment attenuates the intensity of PCNA and caldesmon staining. (B and E) PH also increased the colocalization of PCNA-positive cells with eNOS as early as 10 days after SU treatment. In the SU 21d group the formation of plexiform lesions sprouting from small pulmonary arteries and characterized by severe endothelial cell proliferation (as confirmed by intensive eNOS staining) was detected.

Bosentan treatment attenuates the intensity of PCNA and eNOS staining. (C and E) The development of PH increases the number of CD34-positive cells, which colocalize with PCNA staining. Bosentan treatment attenuates PCNA and CD34 staining and colocalization. (D and E) To confirm that the CD34 signal comes from endothelial cells we colocalized CD34 staining with that of eNOS. PH increases the number of CD34-positive cells that colocalize with eNOS. Bosentan treatment attenuates CD34 and eNOS staining and colocalization. Representative images for each group and each staining are shown from 5–16 pulmonary arteries. The original magnification is $400\times$ and the marker corresponds to 50 μM . $*P < 0.05$ vs control group; $\dagger P < 0.05$ vs SU 21d group.

Table 1

The effect of bosentan on right-ventricle performance and mean systolic blood pressure.

Treatment	RVPSP (mm Hg)	Mean RVP (mm Hg)	RVPDP (mm Hg)	+dP/dt (mm Hg/s)	-dP/dt (mm Hg/s)	Heart rate (b/s)	Mean BP (mm Hg)
Control	29.4 ± 2.3	9.3 ± 1.2	-3.6 ± 1.1	2248 ± 217	-1997 ± 230	400 ± 10	119 ± 4
SU 10d	65.9 ± 6.5 ^a	24.1 ± 2.9 [*]	-1.9 ± 0.7	3810 ± 405 [*]	-3897 ± 362 [*]	400 ± 12	127 ± 7
SU 21d	90.1 ± 5.2 [*]	35.0 ± 3.3 ^{*,a}	-2 ± 0.9	4199 ± 350 [*]	-4141 ± 273 [*]	368 ± 13	134 ± 5
Bosentan	56.1 ± 8.8 ^{*,a}	19.9 ± 3.4 ^{*,a}	-4.1 ± 1.2	3496 ± 562 [*]	-3237 ± 592	420 ± 20	133 ± 11
[*] 1F-ANOVA	<i>P</i> < 0.0058	<i>P</i> < 0.007	<i>P</i> < 0.88	<i>P</i> < 0.04	<i>P</i> < 0.0008	<i>P</i> < 0.99	<i>P</i> < 0.043
^a 1F-ANOVA	<i>P</i> < 0.0208	<i>P</i> < 0.034	<i>P</i> < 0.346	<i>P</i> < 0.51	<i>P</i> < 0.628	<i>P</i> < 0.186	<i>P</i> < 0.926

Results are expressed as means ± SEM; *n* = 8 in each group. Combination of SU5416 and chronic hypoxia induced a severe increase in RV peak systolic pressure (RVPSP) and mild increase in systemic blood pressure (BP), whereas bosentan treatment prevented the progression of pulmonary and systemic hypertension. RVP, RV pressure; RVPDP, RV peak diastolic pressure.

^{*} *P* < 0.05 vs control group.

^a *P* < 0.05 vs SU 21d group.