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RESEARCH PAPER

Intrinsic defence capacity and therapeutic potential of natriuretic peptides in pulmonary hypertension associated with lung fibrosis

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BACKGROUND AND PURPOSE

Idiopathic pulmonary fibrosis (IPF) is a progressive fibro-proliferative disorder refractory to current therapy commonly complicated by the development of pulmonary hypertension (PH); the associated morbidity and mortality are substantial. Natriuretic peptides possess vasodilator and anti-fibrotic actions, and pharmacological augmentation of their bioactivity ameliorates renal and myocardial fibrosis. Here, we investigated whether natriuretic peptides possess an intrinsic cytoprotective function preventing the development of pulmonary fibrosis and associated PH, and whether therapeutics targeting natriuretic peptide signalling demonstrate efficacy in this life-threatening disorder.

EXPERIMENTAL APPROACH

Pulmonary haemodynamics, right ventricular function and markers of lung fibrosis were determined in wild-type (WT) and natriuretic peptide receptor (NPR)-A knockout (KO) mice exposed to bleomycin (1 mg·kg⁻¹). Human myofibroblast differentiation was studied *in vitro*.

KEY RESULTS

Exacerbated cardiac, vascular and fibrotic pathology was observed in NPR-A KO animals, compared with WT mice, exposed to bleomycin. Treatment with a drug combination that raised circulating natriuretic peptide levels (ecadotril) and potentiated natriuretic peptide-dependent signalling (sildenafil) reduced indices of disease progression, whether administered prophylactically or to animals with established lung disease. This positive pharmacodynamic effect was diminished in NPR-A KO mice. Atrial natriuretic peptide and sildenafil synergistically reduced TGFβ-induced human myofibroblast differentiation, a key driver of remodelling in IPF patients.

CONCLUSIONS AND IMPLICATIONS

These data highlight an endogenous host-defence capacity of natriuretic peptides in lung fibrosis and PH. A combination of ecadotril and sildenafil reversed the pulmonary haemodynamic aberrations and remodelling that characterize the disease, advocating therapeutic manipulation of natriuretic peptide bioactivity in patients with IPF.

Abbreviations

α SMA, α smooth muscle actin; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; ILD, interstitial lung disease; iNOS, inducible NO synthase; IPF, idiopathic pulmonary fibrosis; MABP, mean arterial blood pressure; NEPi, neutral endopeptidase inhibitor; NPR, natriuretic peptide receptor; PDE5i, phosphodiesterase 5 inhibitor; PH, pulmonary hypertension; RVH, right ventricular hypertrophy; RVSP, right ventricular systolic pressure; WT, wild-type

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal lung disorder resulting from unrelenting matrix deposition and diminution of lung function (Bjoraker *et al.*, 1998; Eickelberg and Selman, 2010). IPF is the most common interstitial lung disease (ILD) and carries the worst prognosis, with median survival ranging from 2 to 3 years (Martinez *et al.*, 2005; Eickelberg and Selman, 2010; Ley *et al.*, 2011). Pulmonary hypertension (PH), characterized by increased pulmonary vascular resistance, remodelling of the small pulmonary arteries and right ventricular hypertrophy (RVH), is a common complication in IPF (affecting 30–40% of patients), and individuals with a dual diagnosis exhibit markedly reduced survival (Lettieri *et al.*, 2006; Mejia *et al.*, 2009). PH is also frequently associated with other ILDs, including systemic sclerosis-driven pulmonary fibrosis, where it also results in increased mortality (Behr and Ryu, 2008; Condliffe *et al.*, 2009). IPF is refractory to virtually all current therapeutic options and the recently licensed anti-fibrotic, anti-inflammatory drug pirfenidone achieves only a small treatment benefit at high financial cost (Noble *et al.*, 2011). Therefore, identification of novel mechanisms underpinning pathogenesis and potential therapeutic targets that possess the ability to combat both the altered pulmonary haemodynamics and fibro-proliferative lung phenotype is of high priority.

Natriuretic peptides are a family of vasodilator hormones that play a pivotal role in the regulation of blood volume and blood pressure (Lee and Burnett Jr, 2007; Potter *et al.*, 2009). Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are released predominantly from the heart, while a third member of this family, C-type natriuretic peptide, is released principally from endothelial cells. These peptides act via guanylyl cyclase-linked receptors to promote cGMP production, salt and water excretion, and peripheral vasodilation. Natriuretic peptides and their cognate receptors have also been localized to many areas of the lung, including peripheral lung tissues, airway epithelium and pulmonary vasculature (Bianchi *et al.*, 1985; Sakamoto *et al.*, 1986; Gutkowska *et al.*, 1987; Toshimori *et al.*, 1988; receptor nomenclature follows Alexander *et al.*, 2013), and are thought to play a key role in lung physiology, regulating several processes, including vasodilation, bronchorelaxation, pulmonary permeability and surfactant production (Hulks *et al.*, 1990; Perreault and Gutkowska, 1995).

We have recently reported that augmentation of endogenous natriuretic peptide bioactivity, using ecadotril, an inhibitor of neutral endopeptidase [NEPi; an enzyme that metabolizes and inactivates natriuretic peptides (Kenny and Stephenson, 1988; Soleilhac *et al.*, 1992)] in tandem with sildenafil, an inhibitor of phosphodiesterase 5 [PDE5i, hydro-

lyses and inactivates cGMP (Bender and Beavo, 2006)] and first-line treatment of PH, prevents and reverses pathogenesis in a hypoxic model of PH (Baliga *et al.*, 2008). The natriuretic peptide family is also known to exert anti-proliferative and anti-fibrotic bioactivity in the heart and kidney (Tamura *et al.*, 2000; Knowles *et al.*, 2001). Here, we have used a well-characterized model of lung fibrosis induced by bleomycin, to establish a role for endogenous natriuretic peptides in curbing pathogenesis, and the therapeutic potential of this combination of NEPi PDEi in reversing the haemodynamic dysfunction and fibrotic processes that underpin lung fibrosis.

Materials and methods

Bleomycin-induced lung fibrosis

All animal care and experimental studies conformed to the UK Animals (Scientific Procedures) Act 1986 and were approved by the appropriate ethical committee of Queen Mary University of London. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 160 animals were used in the experiments described here.

Mice were housed in a pathogen-free facility with access to food and water *ad libitum*. Littermate wild-type (WT) and natriuretic peptide receptor (NPR)-A knockout (KO) mice (C57B6; male; 20–25 g; kind gift of O. Smithies, University of North Carolina) were exposed to bleomycin (50 μ L \cdot mouse $^{-1}$; 1 mg \cdot kg $^{-1}$ = ~25 IU \cdot per mouse) by oropharyngeal instillation under light isoflurane anaesthesia. Controls were similarly instilled with 50 μ L of sterile saline. Animals were randomly assigned to the following groups:

- Control: saline-treated and receiving daily gavage with vehicle for ecadotril (0.3 mL). This vehicle consists of 0.1% polyethylene glycol + 0.5% carboxymethyl cellulose in water.
- Bleomycin: bleomycin-treated and receiving daily gavage with vehicle
- Bleomycin + sildenafil: bleomycin-treated and receiving sildenafil (30 mg \cdot kg $^{-1}$ in drinking water) and daily gavage of vehicle
- Bleomycin + ecadotril: bleomycin-treated and receiving daily gavage of ecadotril (60 mg \cdot kg $^{-1}$ in vehicle)
- Bleomycin + sildenafil + ecadotril: bleomycin-treated and receiving sildenafil (30 mg \cdot kg $^{-1}$ in drinking water) plus daily gavage of ecadotril (60 mg \cdot kg $^{-1}$ in vehicle)

Treatment was started concomitantly with the administration of bleomycin (i.e. day 0) and outcome measures assessed at day 14. Doses of sildenafil and ecadotril were

chosen based on previous work (Stasch *et al.*, 1995; Zhao *et al.*, 2001; Baliga *et al.*, 2008).

In further experiments, to determine the ability of natriuretic peptides to reverse existing pulmonary fibrosis, mice were exposed to bleomycin (as above), with treatment initiated at day 14 and analysis undertaken at day 28. This 'therapeutic dosing schedule' was chosen after thorough, temporal evaluation of the pathogenesis of the bleomycin model in our laboratory (Scotton and Chambers, 2010), ensuring the progression of established fibrosis was targeted.

In vivo haemodynamic and morphological analysis

Animals were anaesthetized using isoflurane (1.5%, 0.2 mL·min⁻¹ oxygen), and right ventricular systolic pressure (RVSP) and mean arterial blood pressure (MABP) were determined as described previously (Baliga *et al.*, 2008). Following haemodynamic measurements, animals were killed by exsanguination (under isoflurane anaesthesia, as above), hearts excised, and right ventricle to left ventricle plus septum [RV/(LV + S)] ratio determined as a measure of RVH.

Tissue processing and histochemistry

Inflated lungs were fixed in 4% paraformaldehyde for 4 h at 4°C and then incubated overnight in 15% sucrose in PBS at 4°C. Tissue was dehydrated in 70% ethanol and embedded in paraffin. Serial sections (4 µm) were used for MSB (Martius/Scarlet/Blue; DAKO, Ely, UK) staining and α -smooth muscle actin (α SMA) immunohistochemistry. For the latter, sections were incubated with a mouse monoclonal anti- α SMA antibody (DAKO; 1:1000 dilution), followed by biotinylated anti-mouse secondary antibody. Immunoreactivity was detected using the ABC-peroxide based system (DAKO) following the manufacturer's protocol. Stained slides were imaged by Nano-zoomer virtual microscopy (Hamamatsu, Welwyn Garden City, UK). Pulmonary arterial muscularization was then assessed as previously described (Baliga *et al.*, 2008). Briefly, vessels were defined according to the presence or absence of α SMA positive staining. Twenty five muscularized arteries from different fields were imaged at 400 × magnification by light microscopy from representative animals in each group to determine wall thickness. All histology slides were coded and evaluated without knowledge of the treatments.

Total lung collagen analysis

Collagen content was determined by measuring hydroxyproline by reverse-phase HPLC of 7-chloro-4-nitrobenzo-oxao-1,3,-diazole-derived acid hydrolysates; the total lung collagen was then calculated in mg, assuming lung collagen contains 12.2% (w/w) hydroxyproline (Scotton *et al.*, 2009).

Human fibroblast differentiation

The ability of ANP (1 µM) and sildenafil (3 µM) alone, or in combination, to prevent TGF β -driven (1 ng·mL⁻¹) human lung fibroblast differentiation was evaluated as we have described previously using primary human lung fibroblasts isolated from macroscopically healthy segments of lung from patients undergoing lung cancer resection (kind gift of Dr. R.J. McNulty, University College London) (Scotton *et al.*, 2009).

Table 1

Primer sequences (5'→3') used to quantify lung mRNA expression

GENE	Primer sequence
α SMA	Forward: AGAGTGGAGAAGCCCAGCCAGT Reverse: CCAGAGCCATTGTCCGACACCA
TGF β	Forward: GGATACCAACTATTGCTTCAGCTCC Reverse: AGGCTCCAATATAGGGGCAGGGTC
IL-1 β	Forward: GACCTTCCAGGATGAGGACA Reverse: CTAATGGGAACGTCACACACC
IL-13	Forward: CCTGGCTCTTGCTTGCCTT Reverse: GGTCTTGTGTGATGTTTGCTCA
TNF α	Forward: CAAATGGCCTCCCTCTCAT Reverse: CACTTGGTGGTTTGCTACGA

Quantitative real-time polymerase chain reaction for pro-proliferative markers

The expression of mRNA for several pro-proliferative biomarkers was conducted as we have described previously (Scotton *et al.*, 2009). Primers for inducible nitric oxide synthase (iNOS), collagen (Col)-1a and β -actin were from Primer Design (Southampton, UK); the others are shown in Table 1.

Biochemical analyses

Plasma ANP (Phoenix Pharmaceuticals, Karlsruhe, Germany) and cGMP (GE Healthcare, Hatfield, UK) concentrations were measured using commercially available ELISA.

Data analysis

Results are expressed as means \pm SEM. Data were analysed by one-way ANOVA with a Bonferroni post test where appropriate. A Shapiro–Wilk test was applied to data sets to confirm normal distribution. $P < 0.05$ denotes significance.

Materials

Bleomycin was supplied by Kyowa Hakko Kirin Co. Ltd, Tokyo, Japan; ecadotril was a kind gift of Dr. Johannes-Peter Stasch, Bayer AG, Wuppertal, Germany. TGF β was from R&D Systems, Oxford, UK; ANP was supplied by Cambridge Bioscience, Cambridge, UK; isoflurane was from Abbott Laboratories, Maidenhead, UK. Sildenafil was extracted from tablets (Viagra, Pfizer, UK; dispensed by the UCLH pharmacy) by crushing and dissolving in warm (40°C) water, filtration and then acidification with HCl. Extraction was achieved with ethyl acetate to give the free base. The citrate was regenerated from the free base by addition of one equivalent of citric acid and warming in water followed by freeze drying. The drinking water containing sildenafil was replaced with fresh solution every 48 hours.

Results

Effect of sildenafil and ecadotril on haemodynamic and cardiac parameters

Instillation of bleomycin markedly elevated RVSP compared with saline-treated controls (32.0 \pm 5.9 mmHg vs. 21.2 \pm

5.9 mmHg; $P < 0.05$). As monotherapy, neither sildenafil nor ecadotril produced a significant reduction in RVSP in mice receiving bleomycin (Figure 1A). However, the combination of sildenafil plus ecadotril caused a significant fall in RVSP when compared with bleomycin-treated mice. Indeed, the effect of combination therapy was so substantial it virtually reduced RVSP to control (saline-treated) levels (~20 mmHg).

Neither sildenafil nor ecadotril, either alone or in combination, caused a significant change in MABP (Figure 1B).

Bleomycin-treated animals showed a significant increase in RV/(LV + S) ratio, confirming the development of RVH in this model. Treatment with sildenafil or ecadotril alone significantly reduced the bleomycin-induced RVH. However, combination treatment with sildenafil plus ecadotril caused

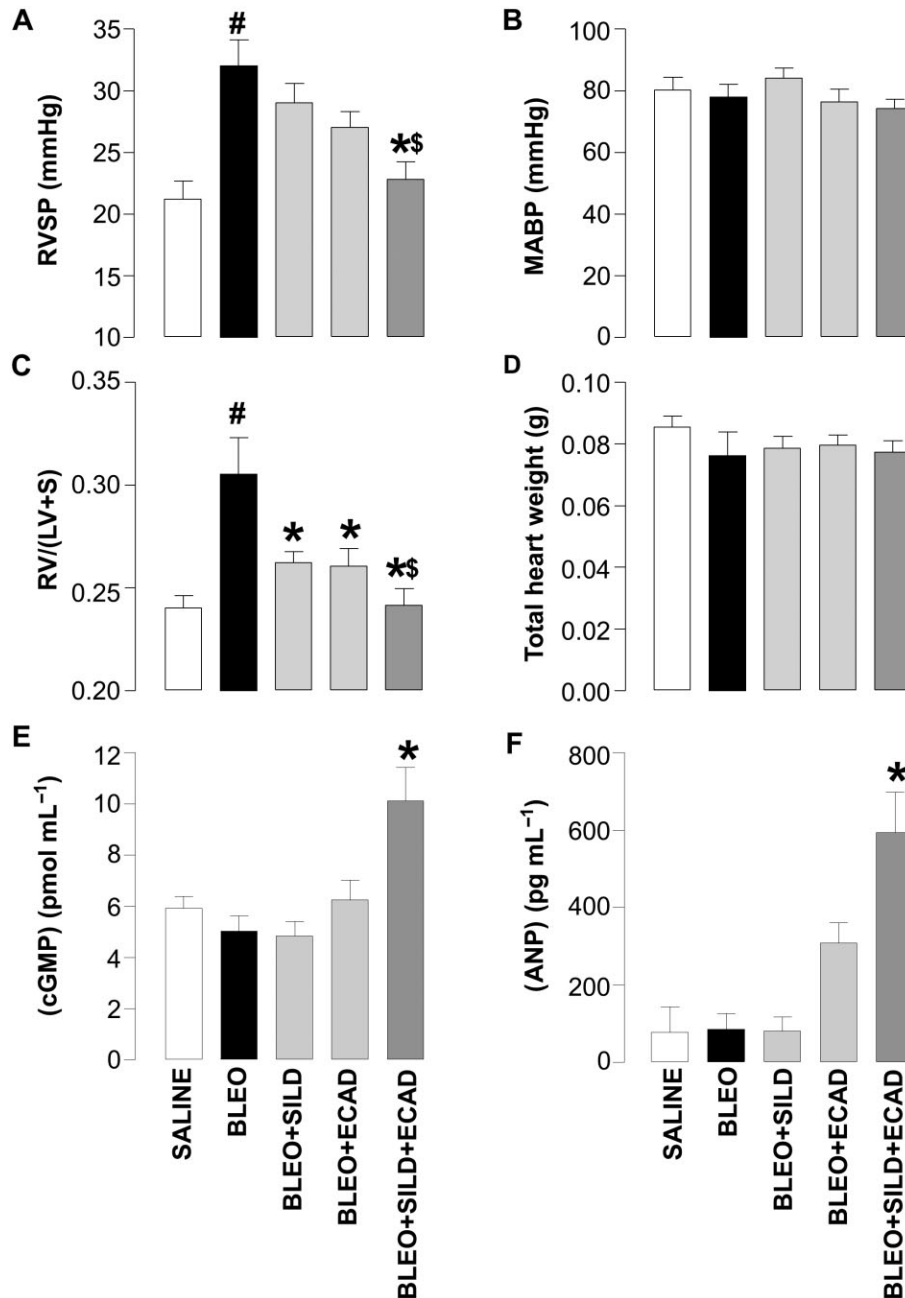


Figure 1

Right ventricular systolic pressure (RVSP) (A), mean arterial blood pressure (MABP) (B), right ventricle : left ventricle plus septum ratio [RV/(LV + S)] (C), total heart weights (D), plasma (cGMP) (E), and plasma (ANP) (F) in saline-treated (control) mice and animals 14 days after exposure to bleomycin (BLEO; 1 mg·kg⁻¹) in the absence and presence of sildenafil (SILD; 30 mg·kg⁻¹·day⁻¹), ecadotril (ECAD; 60 mg·kg⁻¹·day⁻¹) or sildenafil plus ecadotril (doses as above). [#] $P < 0.05$ versus saline control; ^{*} $P < 0.05$ versus bleomycin; ^{\$} $P < 0.05$ versus bleomycin in the presence of sildenafil or ecadotril monotherapy. $n = 17-25$ animals in each group for the haemodynamic measurements, $n = 3-6$ animals for the cGMP and ANP concentrations.

an additional reduction in RVH (Figure 1C). There were no significant changes in total heart weight (Figure 1D).

Changes in pulmonary haemodynamics were mirrored by the concentrations of cGMP and ANP in the plasma, which were only significantly increased in the presence of combination therapy (Figure 1E and F).

Effect of sildenafil and ecadotril on pulmonary vascular remodelling

Control animals showed only a modest degree of pulmonary muscularization, which was significantly increased by bleomycin (Figure 2A). Treatment with sildenafil, but not ecadotril ($P = 0.07$ vs. bleomycin), caused significant attenuation of this bleomycin-induced muscularization. However, the combination of sildenafil plus ecadotril produced a significantly larger decrease in the percentage of muscularized arteries compared with either treatment alone (Figure 2B). An essen-

tially identical pattern of activity was observed with respect to vessel wall thickness (difference between the internal and external diameter of α SMA-stained vessels (Figure 2C), although in this instance ecadotril alone produced a significant salutary effect on wall thickness.

Effect of sildenafil and ecadotril on lung fibrosis

Bleomycin-injured animals exhibited typical fibrotic lesions consisting of increased cellularity (including fibroblasts) and deposition of extracellular matrix. Mice receiving combination treatment following bleomycin administration showed a clear reduction in the incidence of fibrotic damage (Figure 3A).

Bleomycin administration caused a significant increase in total lung weight (Figure 3B), which was reduced in the presence of sildenafil, ecadotril or the combination (Figure 3B). In

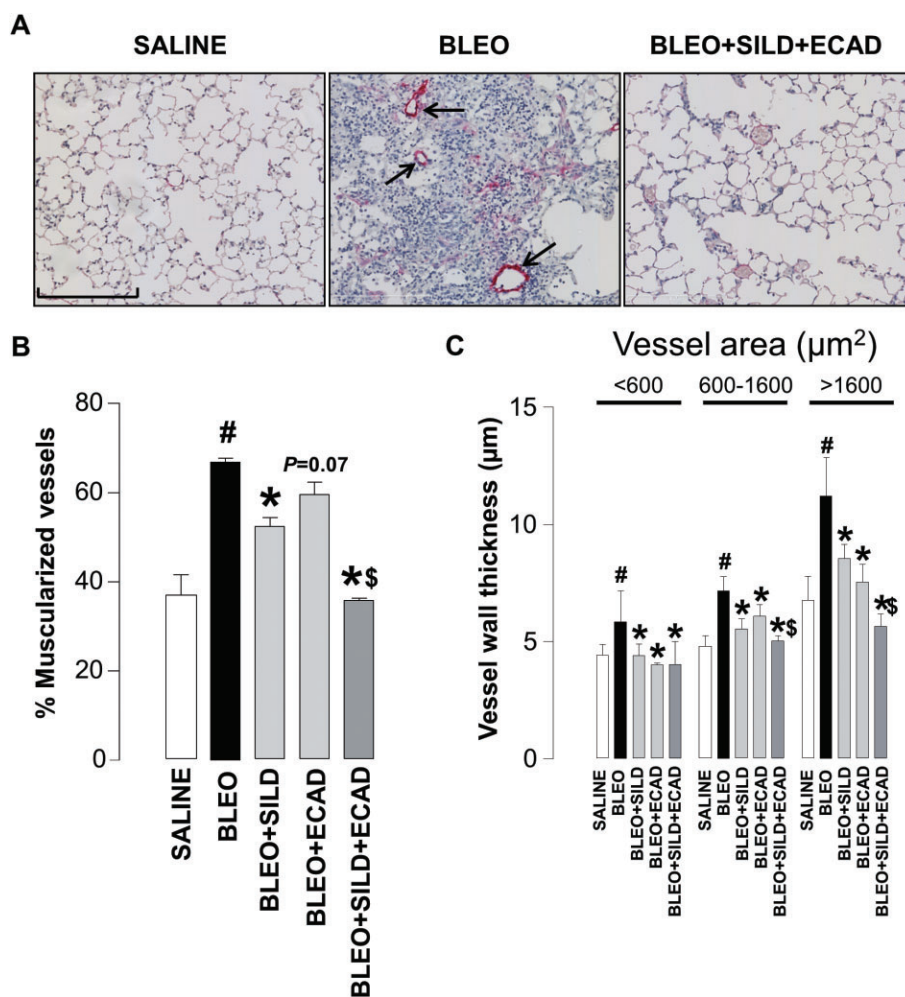


Figure 2

Representative light microscopic images (scale bar = 200 µm) (A), and quantitative assessment of muscularization (B) and wall thickness (C) of pulmonary arteries from saline controls, bleomycin (BLEO; 1 mg·kg⁻¹)-treated animals, and mice receiving bleomycin in the presence of sildenafil (SILD; 30 mg·kg⁻¹·day⁻¹), ecadotril (ECAD; 60 mg·kg⁻¹·da⁻¹) or sildenafil plus ecadotril (doses as above). Arrows indicate areas of overt muscularization (α SMA staining). [#] $P < 0.05$ versus saline control; ^{*} $P < 0.05$ versus bleomycin; ^{\$} $P < 0.05$ versus bleomycin in the presence of sildenafil or ecadotril monotherapy. $n = 17$ –25 animals in each group.

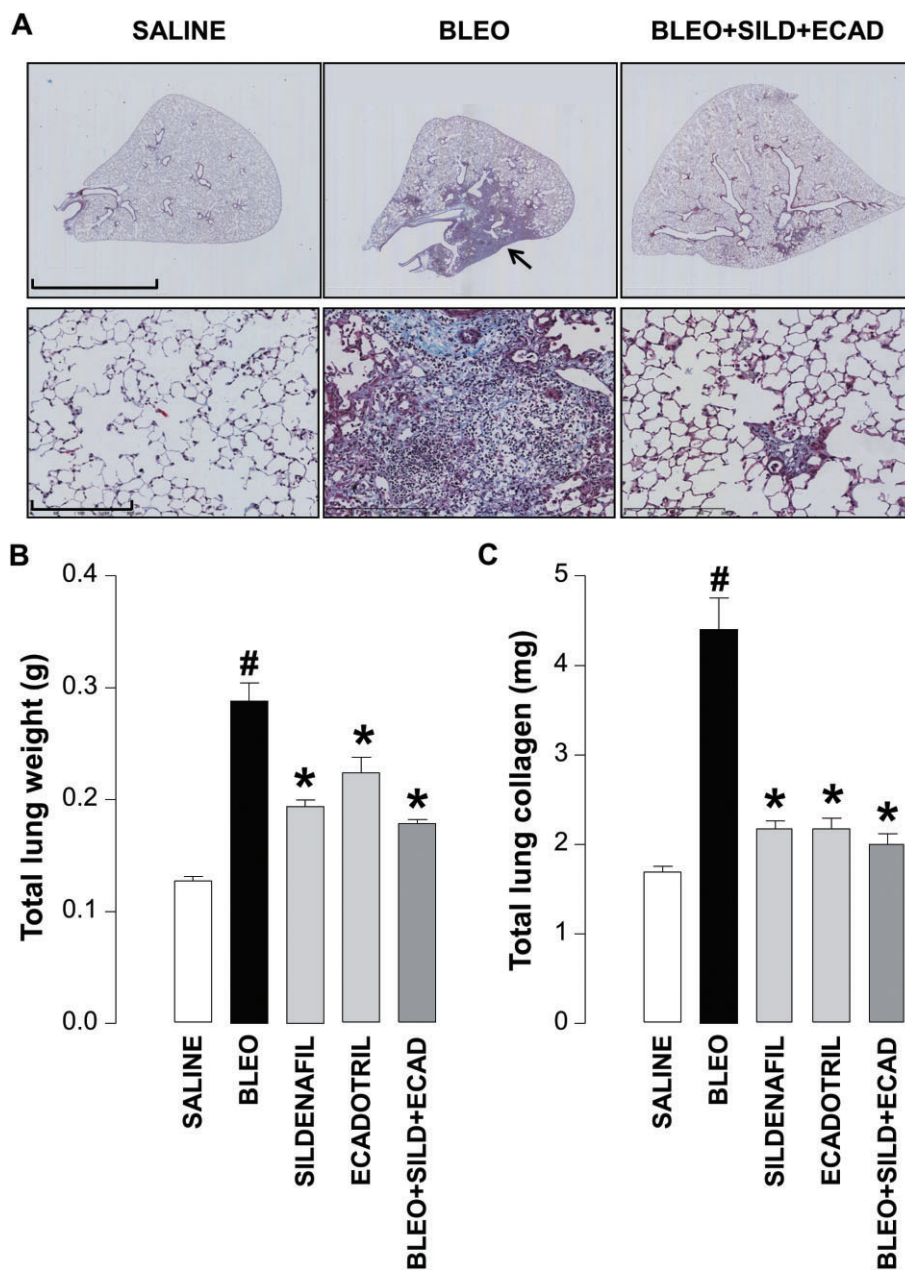


Figure 3

Representative light microscopic images of whole lung cross-sections (upper panels; scale bar = 4 mm) and higher magnification of the same samples (lower panels; scale bar = 200 μm) stained with Martius/Scarlet/Blue (MSB) (A), and quantitative assessment of lung weight (B) and total lung collagen (C) in saline controls, bleomycin (BLEO; $1 \text{ mg}\cdot\text{kg}^{-1}$)-treated animals, and mice receiving bleomycin in the absence and presence of sildenafil (SILD; $30 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$), ecadotril (ECAD; $60 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or sildenafil plus ecadotril (doses as above). Arrows indicate areas of overt fibrosis. [#] $P < 0.05$ versus saline control; ^{*} $P < 0.05$ versus bleomycin. $n = 17\text{--}25$ animals in each group.

accord, bleomycin induced a marked increase in the total lung collagen content that was also attenuated by monotherapy or dual therapy (Figure 3C). In this case, the significant salutary effects of sildenafil and ecadotril *per se* entailed any supplementary advantage with combination treatment was negligible. Nonetheless, the effect of each treatment on collagen content was remarkable, with >80% attenuation in each case.

Effect of sildenafil and ecadotril on the expression of pro-fibrotic, pro-inflammatory markers in the lung

To provide additional evidence that the combination therapy exerted an anti-fibrotic effect in this model, we determined the expression of mediators known to play a role in the pathogenesis of IPF, including TGF β , IL-1 β , IL-13 and iNOS.

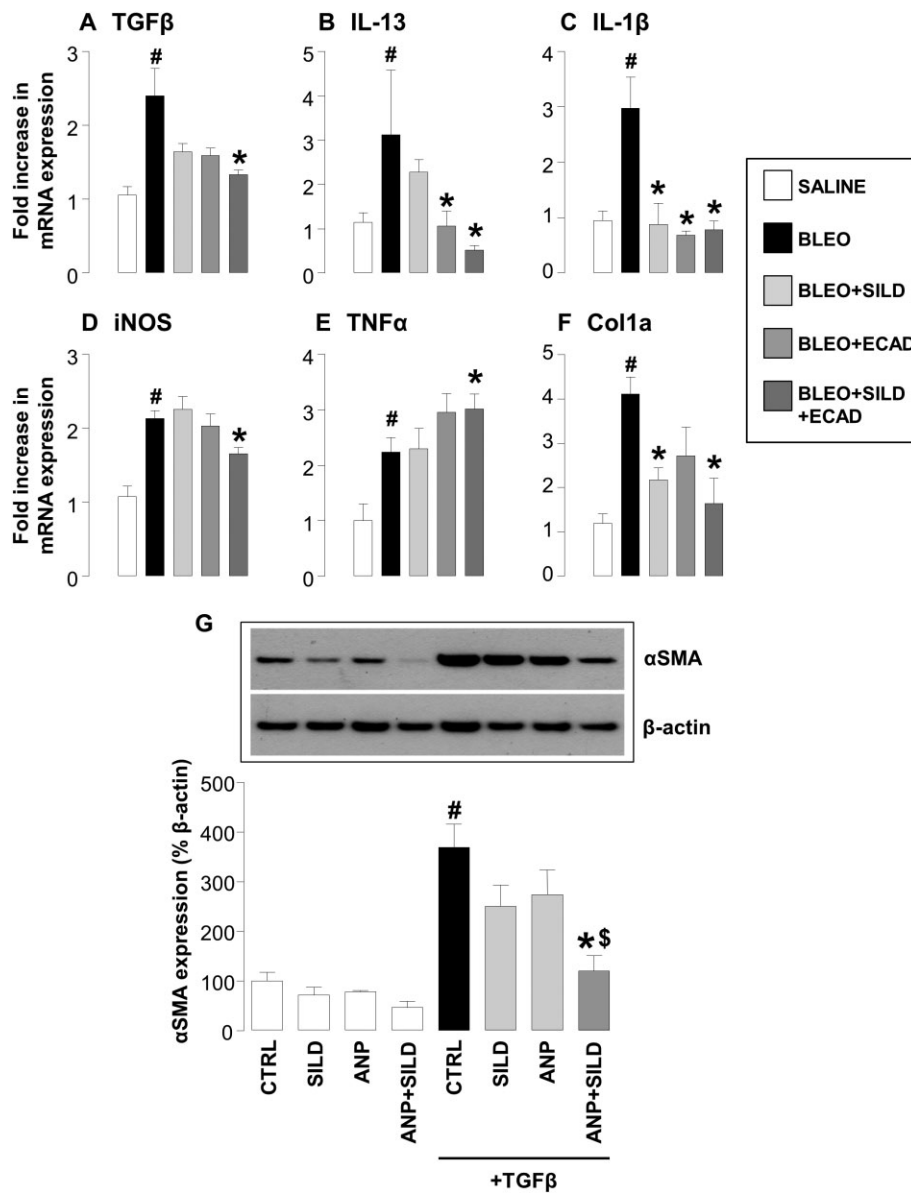


Figure 4

(A–F) Expression of mRNA encoding classical pro-fibrotic/pro-inflammatory mediators in the lungs of saline controls, bleomycin (BLEO; 1 mg·kg⁻¹)-treated animals, and mice receiving bleomycin in the presence of sildenafil (SILD; 30 mg·kg⁻¹·day⁻¹), ecadotril (ECAD; 60 mg·kg⁻¹·day⁻¹) or sildenafil plus ecadotril (doses as above). [#]*P* < 0.05 versus saline control; ^{*}*P* < 0.05 versus bleomycin. *n* = 9 experiments for each group. (G) Expression of α smooth muscle actin (α SMA) by human fibroblasts under control conditions or stimulated with TGF β (1 ng·mL⁻¹) in the absence and presence of sildenafil (3 μ M), atrial natriuretic peptide (ANP; 1 μ M) or sildenafil plus ANP (at the same concentrations). [#]*P* < 0.05 versus saline control; ^{*}*P* < 0.05 versus TGF β ; ^{\$}*P* < 0.05 versus TGF β in the presence of sildenafil or ecadotril monotherapy. *n* = 9 experiments for each group.

In addition, we assessed expression of mRNA for Col1a to confirm the anti-fibrotic activity of combination therapy observed *in vivo* affected collagen production at a transcriptional level.

Bleomycin treatment resulted in a significant increase in the expression of these mediators, which in accord with effects on collagen deposition was significantly reduced by combination treatment. Monotherapy did reduce the mRNA expression for some mediators (e.g. ecadotril lowered the expression of IL-13), but neither sildenafil nor ecadotril alone

was able to produce the wide-ranging inhibitory effect on pro-fibrotic/pro-inflammatory pathways that dual therapy delivered (Figure 4A–D and F). Intriguingly, the expression of TNF α was enhanced by the combination treatment (Figure 4E).

Effect of sildenafil and natriuretic peptides on human fibroblast differentiation in vitro

One of the key steps in the development of pulmonary fibrosis is the differentiation of fibroblasts into α SMA-expressing

myofibroblasts (driven principally by TGF β) that secrete a number of pro-proliferative factors, and are the major source of collagen and extracellular matrix (Burgess *et al.*, 2005; Scotton and Chambers, 2007) that precipitate remodelling. To demonstrate that combination therapy was effective in inhibiting this pivotal step in the pathogenesis of fibrosis, and to provide a rudimentary proof of concept in human cells, we explored the ability of natriuretic peptides and PDE5i to block human fibroblast differentiation *in vitro*.

TGF β caused a rapid differentiation of human lung fibroblasts into myofibroblasts as quantified by the appearance of α SMA positive cells (Figure 4G). In the presence of ANP or sildenafil alone, there was a subtle decrease in the degree of α SMA accumulation. However, the combination of ANP plus sildenafil caused a synergistic inhibition of α SMA up-regulation and fibroblast–myofibroblast differentiation (Figure 4G).

Effect of NPR-A gene deletion on the development of PH and fibrosis

In order to establish a role for endogenous natriuretic peptides in limiting pathogenesis in IPF, and to confirm that the beneficial effects of combination therapy are dependent on natriuretic peptide bioactivity, we repeated representative studies in NPR-A KO animals. These mice lack the guanylyl cyclase-linked receptor that acts as the principal target for ANP and BNP (Potter *et al.*, 2009). In these animals, administration of bleomycin dramatically increased RVSP beyond that observed in WT animals (Figure 5A), suggesting that endogenous natriuretic peptides act in an intrinsic defence capacity. While NPR-A KO animals exhibited a higher MABP, this was not significantly altered by sildenafil, ecdotril or the combination in WT or NPR-A KO mice (Figure 5B). Bleomycin also aggravated RVH in NPR-A KO animals, although this is masked somewhat by the inherent left heart hypertrophy characteristic of this KO strain. Importantly, the beneficial effects of the combination therapy (sildenafil plus ecdotril) were diminished in NPR-A KO mice (Figure 5C).

A similar profile of activity was observed with respect to lung fibrosis. Bleomycin caused an increase in total lung weight that was greater than that reached in WT animals (Figure 5D). Nonetheless, the effect of combination treatment to block the increase in lung weight was blunted in the NPR-A KO mice (Figure 5D).

Effect of sildenafil and ecdotril on established PH and lung disease

To more closely parallel the clinical situation, additional investigations were conducted in which combination therapy was initiated 2 weeks post-bleomycin, a so-called therapeutic dosing regimen (Scotton and Chambers, 2010). In this case, bleomycin caused the expected increase in RVSP, RVH, total lung collagen and fibrosis (Figure 6A, B, D and E). Despite the delayed administration of treatment, the combination of sildenafil and ecdotril remained effective in reducing each of these parameters (Figure 6A, B, D and E). As with prophylactic treatment, neither sildenafil nor ecdotril, alone or in combination, caused a change in MABP (Figure 6C).

Discussion

IPF is a progressive fibro-proliferative disorder with a poor prognosis; this is largely the result of a complex and undefined aetiology, and a lack of therapeutic options (Bjoraker *et al.*, 1998; Eickelberg and Selman, 2010). ILDs, including IPF, are frequently complicated by the existence of PH; a dual diagnosis that has a far less favourable outcome (Lettieri *et al.*, 2006; Mejia *et al.*, 2009). We reported recently that augmentation of natriuretic peptide bioactivity results in a significant alleviation of disease severity in hypoxia-induced PH (Baliga *et al.*, 2008). This precedent, coupled with the well-defined anti-fibrotic actions of natriuretic peptides in the heart and kidney (Tamura *et al.*, 2000; Soeki *et al.*, 2005; Li *et al.*, 2008; Nishikimi *et al.*, 2009; Das *et al.*, 2010), provided the rationale to explore the potential of manipulating natriuretic peptide bioactivity in IPF. Data presented here suggest that this approach may offer a substantial pharmacodynamic benefit in IPF and associated PH as it combats both the haemodynamic aberrations and fibro-proliferative aspects of the disease.

Adopting a well-validated model of inflammation and lung injury that is accepted as a model of human lung fibrosis (Scotton and Chambers, 2010), we demonstrate that focusing efforts to maximize cGMP-dependent signalling, using PDE5i and NEPi, offers a potent means of preventing and reversing fibrosis and the accompanying PH. Administration of bleomycin caused the expected increase in lung fibrosis, significantly elevated RVSP and promoted RVH. Neither of the components of the combination therapy (i.e. sildenafil or ecdotril) significantly reduced RVSP at the doses employed. However, this provided the ideal background to reveal a clear synergy between the two drugs when administered together; this cooperative activity mirrored that reported in models of hypoxia-induced PH and patients with the disease (Zhao *et al.*, 2003; Preston *et al.*, 2004; Klinger *et al.*, 2006; Baliga *et al.*, 2008). A salutary effect on the right heart was also evident. Interestingly, the effects of the combination therapy were greater (individually and in concert) against structural changes in the right heart compared with the haemodynamic dysfunction. This disparity suggests that augmentation of natriuretic peptide function has direct effects to slow or prevent cardiac hypertrophy, rather than simply secondary to reducing pressure in the pulmonary circulation. This is perhaps not surprising, since all three of the principal members of the natriuretic peptide family exert potent anti-hypertrophic effects in the heart (Tamura *et al.*, 2000; Knowles *et al.*, 2001). This is a welcome finding since the right heart is often neglected in the consideration of novel therapies for IPF and PH, and treatment modalities that directly preserve right heart structure and function are likely to provide a valuable addition to the therapeutic repertoire. A further key facet of this PDE5i plus NEPi combination is that it appears to target the lung, as MABP was not significantly affected. This selectivity is advantageous in IPF and PH since it avoids the issue of systemic hypotension while being able to maximize cGMP signalling in the pulmonary circulation.

In response to increased resistance, the pulmonary circulation remodels to accommodate the higher pressure and maintain oxygenation. Induction of fibrosis with bleomycin

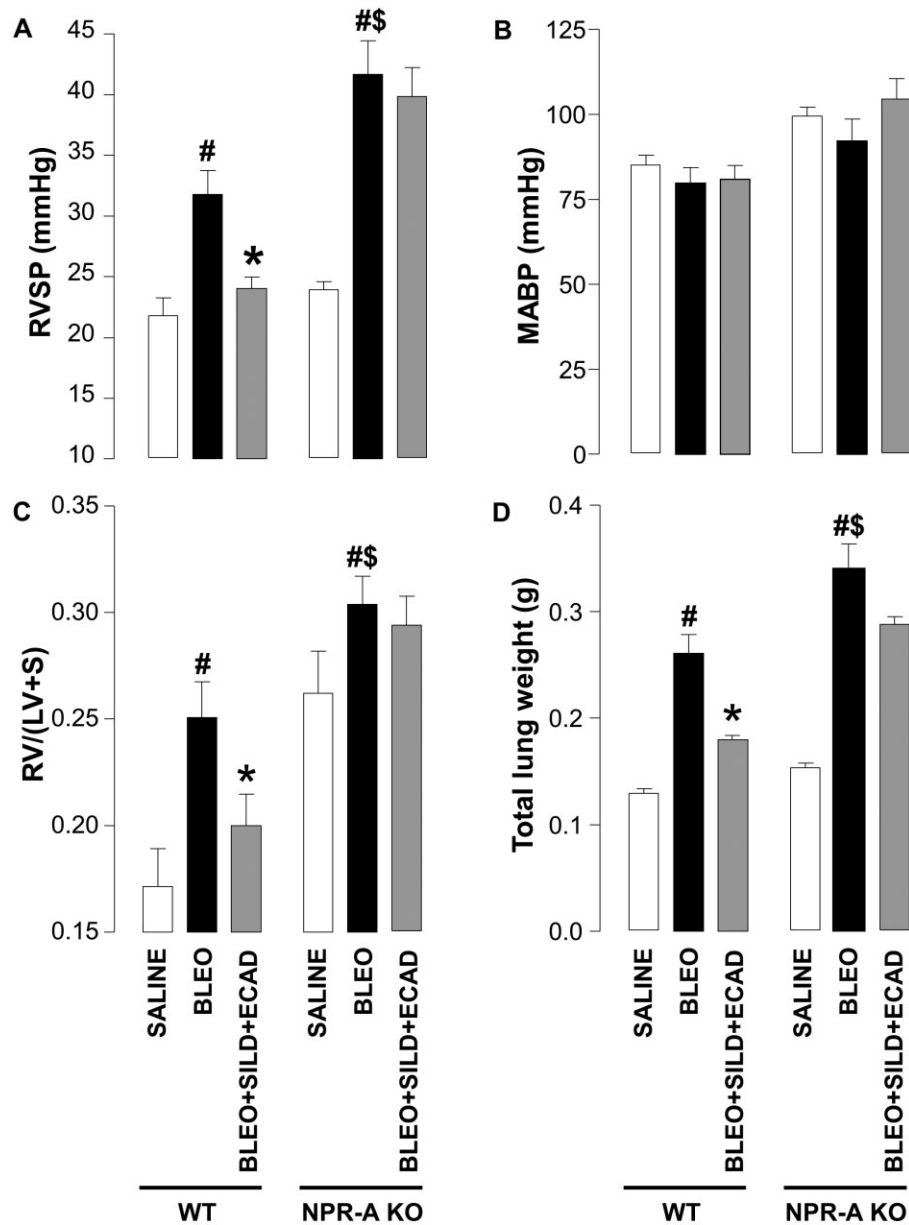


Figure 5

Right ventricular systolic pressure (RVSP) (A), mean arterial blood pressure (MABP) (B), right ventricle : left ventricle plus septum ratio [RV/(LV + S)] (C), and total lung weight (D) in wild-type (WT) mice and natriuretic peptide receptor (NPR)-A KO animals exposed to saline, or bleomycin (BLEO; 1 mg·kg⁻¹) in the absence and presence of sildenafil (SILD; 30 mg·kg⁻¹·day⁻¹) plus ecdotril (ECAD; 60 mg·kg⁻¹·day⁻¹). #*P* < 0.05 versus saline control; **P* < 0.05 versus bleomycin; \$*P* < 0.05 versus bleomycin-treated WT. *n* = 6–12 animals in each group.

also caused a dramatic increase in the number of muscularized small pulmonary arteries. Akin to the positive effect recorded in hypoxia-induced PH (Baliga *et al.*, 2008), here the remodelling of the pulmonary circulation was significantly reduced by monotherapy with PDE5i or NEPi, with a clear additive if not synergistic activity of dual therapy. This provides further evidence that augmenting natriuretic peptide bioactivity exerts a multifaceted beneficial effect on many haemodynamic aspects of pathogenesis in PH linked to IPE. Undoubtedly, if these natriuretic peptide-driven salutary

actions translate to the clinical arena, combination treatment should significantly improve outcome. Indeed, the beneficial pharmacodynamic profile of PDE5i plus NEPi holds promise in the treatment of many forms of PH as it appears that the combination therapy is effective in models of PH with contrasting aetiologies.

Combination therapy also produced an impressive reversal of the fibrotic aspects of lung disease. The outcome of prophylactic treatment was manifested as a marked reduction in the total lung weight, collagen deposition, and diminished

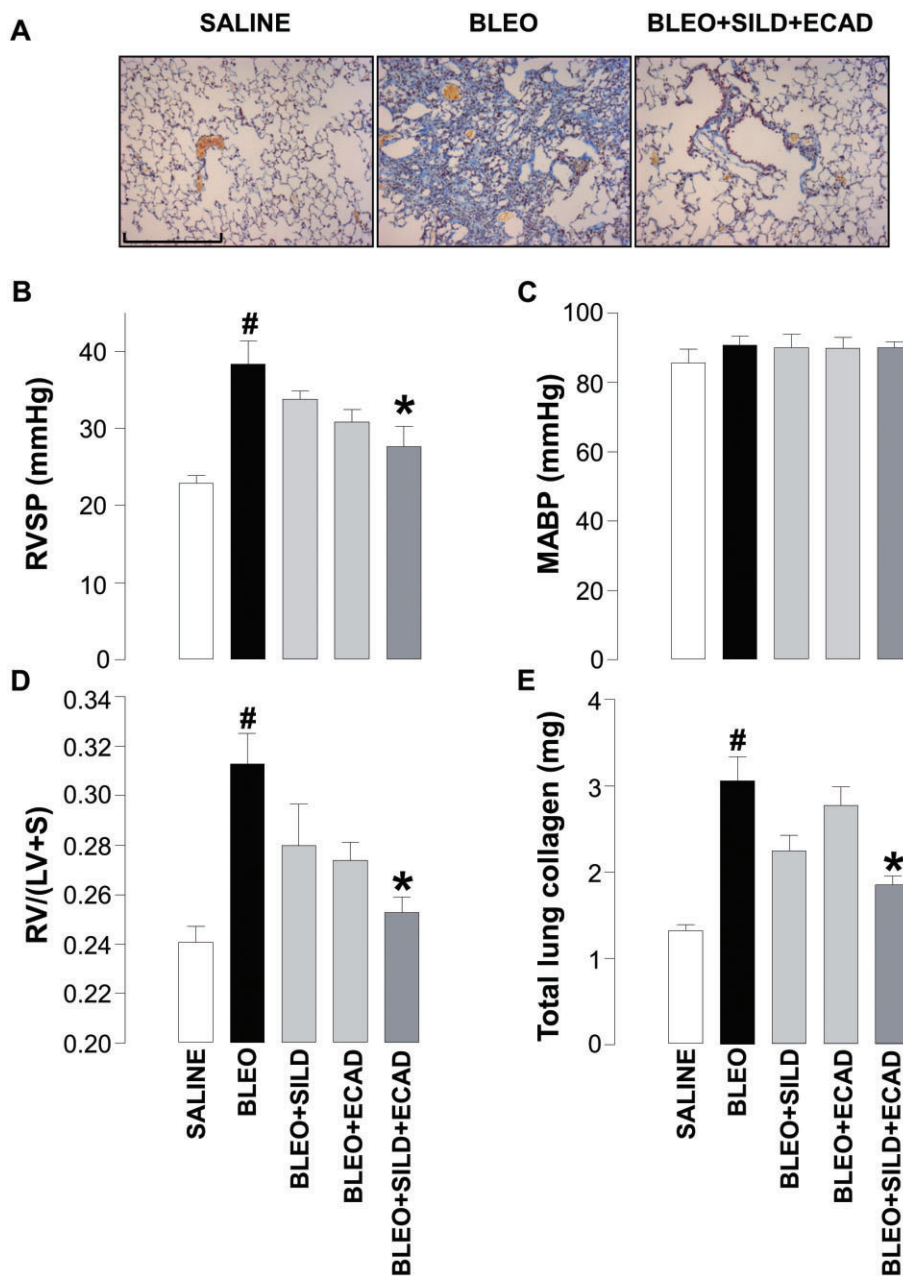


Figure 6

Representative light microscopic images of (scale bar = 200 μm) (A), right ventricular systolic pressure (RVSP) (B), mean arterial blood pressure (MABP) (C), right ventricle : left ventricle plus septum ratio [RV/(LV + S)] (D) and total lung weight (E) in saline controls, bleomycin (BLEO; 1 $\text{mg}\cdot\text{kg}^{-1}$)-treated animals, and mice receiving bleomycin in the presence of sildenafil (SILD; 30 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) plus ecadotril (ECAD; 60 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$). Sildenafil and ecadotril were administered 2 weeks post-bleomycin exposure. [#] $P < 0.05$ versus saline control; ^{*} $P < 0.05$ versus bleomycin. $n = 6$ –10 animals in each group.

expression of a number of classically pro-inflammatory and pro-fibrotic genes (e.g. TGF β , IL-1 β , IL-13) (Maher *et al.*, 2007; Scotton and Chambers, 2010; Farkas *et al.*, 2011) in animals exposed to bleomycin. A major criticism of the bleomycin model is that prophylactic treatment regimens may dampen the inflammatory phase of the response to bleomycin, with a consequent reduction in fibrogenesis (rather than having direct anti-fibrotic efficacy). To address this issue, we also

adopted a ‘therapeutic dosing strategy’, administering therapy once fibrosis was already well established. Our data again showed a remarkable effect of the combination therapy on the increase in RVSP, RVH and total lung weight. Facilitation of cGMP-dependent signalling by combination therapy, therefore, exerts a key restraint on one of the central processes leading to overt pulmonary fibrosis, that of extracellular matrix deposition.

To elucidate the mechanism of this anti-fibrotic action, we investigated the effect of natriuretic peptides on fibroblast differentiation (into highly synthetic and contractile α SMA-expressing myofibroblasts), which underpins the synthesis and deposition of extracellular matrix and is perceived to be a key step in the development of IPF (Burgess *et al.*, 2005; Scotton and Chambers, 2007). In our experiments, ANP and sildenafil showed no significant effect on fibroblast differentiation individually, but in combination almost completely prevented the differentiation. This fits well with the observations that circulating natriuretic peptide levels increase in patients with pulmonary fibrosis (Burghuber *et al.*, 1988; Leuchte *et al.*, 2004), advocating the belief that these traditionally cardioprotective mediators offer an innate defence mechanism against (pulmonary) fibrosis. Indeed, there is considerable precedent to support the thesis that natriuretic peptides might exert a wide spectrum of activities that counteract pulmonary fibrosis, thereby enhancing their credentials as a novel therapeutic strategy. For example, natriuretic peptides can prevent TGF β -induced myofibroblast formation from cardiac fibroblasts, probably by disrupting TGF β -induced nuclear translocation and downstream signalling of pSmad3 (Kapoun *et al.*, 2004). Moreover, in patients with heart failure, TGF β concentrations inversely correlate with NT-proBNP levels (Behnes *et al.*, 2011), and NPR-A KO mice develop renal fibrosis that is associated with increased epithelial-mesenchymal transition and expression of TGF β (Das *et al.*, 2010). Indeed, blockade of Smad signalling appears a common theme in the anti-TGF β effects of natriuretic peptides since this phenomenon is observed in cardiac fibroblasts, proximal tubular cells and pulmonary artery smooth muscle cells (Kapoun *et al.*, 2004; Li *et al.*, 2008; Lo *et al.*, 2008); whether a similar intervention underlies the beneficial effects of combination therapy in pulmonary fibrosis warrants further attention.

Inhibition of the expression and activity of principal pro-inflammatory and pro-fibrotic cytokines critical in the development of pulmonary fibrosis (Gasse *et al.*, 2007; Scotton and Chambers, 2007; 2010) appears another weapon in the anti-fibrotic arsenal of natriuretic peptides. For example, TGF β is perhaps the best characterized and fundamental driver of fibrotic lung disease; it promotes epithelial activation and dysregulation, and facilitates fibroblast differentiation and proliferation leading to collagen production and deposition (Border and Noble, 1994; Willis and Borok, 2007). The Th₂ cytokine IL-13 has been shown to promote fibrosis in a number of experimental models (Wynn, 2003), and IL-13-deficient mice exhibit exacerbated lung fibrosis (Kolodtsick *et al.*, 2004). Inducible NOS also contributes to the development of pulmonary fibrosis and underlies the response to a number of pro-fibrotic cytokines, including IL-5 and IL-13 (Naura *et al.*, 2010). iNOS expression is easily identifiable in the lungs of patients with IPF (Saleh *et al.*, 1997) and is thought to expedite the development of fibrosis (Naura *et al.*, 2010). Each of these classically pro-fibrotic genes showed enhanced expression following bleomycin treatment that was significantly attenuated in mice treated with sildenafil plus ecadotril. This widespread inhibition of the expression of pro-fibrotic genes dovetails well with the favourable effects of combination therapy on the haemodynamic and fibrotic complications of bleomycin exposure, and suggest that phar-

macological augmentation of cGMP signalling has a multi-pronged treatment effect. The mechanism(s) underpinning this broad anti-fibrotic/anti-inflammatory influence of combination therapy warrant further attention, but a similar pattern of activity of natriuretic peptides has been reported in acute lung injury (Koga *et al.*, 2010); one plausible explanation and common pathway is a cGMP-mediated inhibition of NF- κ B activity, perhaps via inhibition of phosphorylation and degradation of the inhibitory subunit I κ B, which has been reported *in vitro* and *in vivo* (Moriyama *et al.*, 2006; Ladetzki-Baehs *et al.*, 2007; Das *et al.*, 2010). TNF α levels were also increased in response to bleomycin, consistent with the belief that this cytokine is involved in the pathogenesis of fibrosis (Piguet *et al.*, 1993; Zhang *et al.*, 1993). Intriguingly, we observed a slight enhancement of TNF α mRNA levels in the bleomycin-treated mice that had received combination therapy. As it was primarily considered a mitogen, anti-TNF α therapies have been evaluated in a variety of fibrotic disorders, but evidence suggests that these approaches may exacerbate disease severity, notably in pulmonary fibrosis (Thavarajah *et al.*, 2009). Our data suggest that TNF α has a net anti-fibrotic effect, at least in the bleomycin experimental model, perhaps due to a pro-apoptotic effect via the TNF receptor-1 (Sun and Fink, 2007).

In the present study, by utilizing NPR-A KO mice, we demonstrate that not only does augmentation of natriuretic peptide signalling represent a tangible means to reduce disease severity, but also that endogenous release of natriuretic peptide represents an intrinsic defence mechanism that offsets the progression of pulmonary fibrosis. Using haemodynamic (e.g. RVSP) and structural (e.g. RVH, total lung weight) indices of pathogenesis, in animals lacking innate natriuretic peptide bioactivity (i.e. NPR-A KO) pathology is exacerbated. In addition, the beneficial effects of PDE5i plus NEPi dual therapy were largely absent in NPR-A KO mice. This finding confirms that the principal mechanism that the dual therapy triggers to prevent or reverse pulmonary fibrosis is augmentation of the bioactivity of ANP and/or BNP. This is a key point since NEP metabolizes a number of vasoactive peptides, such as endothelin-1, bradykinin, and vasoactive intestinal peptide (Campbell, 2003), yet it appears that it is primarily the enhancement of the cytoprotective effects of natriuretic peptides that underpins the efficacy of combination treatment in IPF. This conclusion is supported by the observation that plasma ANP and cGMP levels were only significantly increased by combination therapy.

In summary, a combination therapeutic strategy targeted to enhance natriuretic peptide activity (NEPi) and prevent cGMP catabolism (PDE5i) is a potent and effective combination that prevents and reverses the fibrosis and accompanying PH in bleomycin-induced lung inflammation and injury. Moreover, natriuretic peptides represent an intrinsic cytoprotective pathway that is triggered during the pathogenesis of pulmonary fibrosis and that offsets disease progression. Therefore, this NEPi/PDE5i drug combination might be a novel therapeutic approach in IPF, which currently has little or no disease-modifying therapy, and consequently significant associated morbidity and mortality. As both components of the combination are licensed drugs, efficacy in IPF patients can be evaluated rapidly and inexpensively. This approach,

therefore, shows promise to improve outcome and lower healthcare costs in this patient cohort.

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Author contributions

R.S.B., C.J.S. and S.L.T. conducted the studies and applied statistical analyses. All authors contributed to the study design and drafting of the manuscript.

Conflict of interest

A.J.H. has acted as a consultant/advisory board member for Bayer AG, Novartis, Merck and Palatin Technologies.

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